Biochemical characterization of a type II diacylglycerol acyltransferase from *Claviceps purpurea*

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ABSTRACT

Claviceps purpurea, a fungal pathogen, of ergot diseases in agriculturally important cereal crops, produces high levels of glycerides containing ricinoleic acid (12-hydroxyoctadec-*cis*-9-enoic acid) in its sclerotia. A fatty acid hydroxylase (CpFAH) involved in the biosynthesis of ricinoleic acid was recently identified from *C. purpurea*. This research describes the biochemical characterization of a type II diacylglycerol acyltransferase (CpDGAT2) involved in the assembly of this fatty acid into triglycerides from *C. purpurea*. Expression of CpDGAT2 in a quadruple mutant *Saccharomyses cerevisiae* H1246, in which all four triacylglycerol (TG) biosynthesis genes (*DGA1, LOR1, ACAT1 and ACAT2*) were disrupted, restored the ability of the mutant to synthesize TGs *in vivo*. *In vitro* enzymatic assays of microsomal preparations of the transformants indicated that CpDGAT2 preferentially use ricinoleic acid over linoleic acid, oleic acid and linolenic acids as acyl donor, and 1,2-dioleoyl-*sn*-glycerol over 1,2-dipalmitoyl-*sn*-glycerol as acyl acceptor. CpDGAT2 did not show any activities for the formation of wax esters and estolides when 1-hexadecanol and triricinolein were used as acyl acceptors. Co-expression of CpFAH and CpDGAT2 in yeast resulted in increased accumulation of ricinoleic acids compared to expression of CpFAH along with the yeast native DGAT2 (ScDGA1) or expression of CpFAH alone. Northern blot analysis indicated that *CpFAH* is solely expressed in sclerotium cells and no transcripts of this gene were detected in mycelium and conidium cells. CpDGAT2 is more widely expressed in cell types examined except for conidiospores where the expression is low. The highest expression of CpDGAT2 was detected in 20 day-old sclerotium cells where the highest levels of ricinoleate glycerides are accumulated.

Collectively, these data indicate CpDGAT2 and CpFAH are two key enzymes coordinating the biosynthesis and bioassembly of ricinoleic acid in *C. purpurea.*

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1. INTRODUCTION

The genus *Claviceps* includes specialized fungi which parasitize the flowers of specific grasses without affecting other parts of the plants. *Claviceps* species belong to the family *Clavicipitaceae* with *Claviceps purpurea* as the most representative and studied specie of this genus. *C. purpurea* infects the ovary of the inflorescences of grasses, sedges, and rushes. During its life cycle, *C. purpurea* forms a structure called a sclerotium, in which the fungus survives over the winter. It has long been known that oil extracted from sclerotia of many strains of *Claviceps purpurea* contains a considerable portion of ricinoleic acid (12-hydroxyoctadec-*cis*-9-enoic acid), which in some cases amounts to as much as 50% of the total fatty acids (Batrakov and Tolkacher 1997).

Ricinoleic acid is a hydroxylated fatty acid of particular interest in the oleo-chemical industry. It was believed for long time that the biosynthesis of this fatty acid in *C. purpurea* involved a hydration process, with linoleic acid as the substrate (Morris et al., 1966). However, van de Loo et al., (1995) identified an oleate 12-hydroxylase gene from *Ricinus communis* (RcFAH) which catalyzed a direct hydroxyl substitution of an oleic moiety at the 12C position. A similar oleate 12-hydroxylase (CpFAH) isolated recently by our laboratory from *C. purpurea* also has the ability to incorporate a hydroxyl group in the 12C position of oleic acid (Meesapyodsuk and Qiu, 2008). Currently, the agriculturally important source of ricinoleate is *R. communis*, in which ricinoleic acid the major fatty acid in triacylglycerols (TGs) accounts for up to90% of the total fatty acids. Bafor et al., (1991) investigated the enzymatic substrate preference for the first and second steps in TG synthesis in developing seed of *R. communis* and

found that ricinoleic acid was incorporated in the *sn-1* and *sn-2* positions of TGs. It remains unclear whether the last step of TG biosynthesis also preferentially utilizes ricinoleic acid as an acyl donor substrate.

The last step in TG synthesis is catalyzed by diacylglycerol acyltransferase (DGAT) (Kennedy, 1961), an enzyme which has been well studied due to its association with diseases such as obesity and arteriosclerosis. Two types of DGAT have been identified, namely DGAT1 and DGAT2, which, while related by function, share no homology (Lardizabal et al., 2001). The DGAT2 gene from *R. communis* has previously been characterized in regards to its substrate selectivity (Kroon et al., 2006, Burgal et al., 2008). However, to our knowledge no DGAT2 has been functionally proven to preferentially utilize ricinoleic acid as an acyl donor. In this study we report the biochemical characterization of a *C. purpurea* gene isolated in our lab, which encodes a diacylglycerol acyltransferase type II (CpDGAT2) involved in the preferential assembly of ricinoleate into TG.

1.2 Study rationale

Ricinoleic acid-containing TG has many industrial applications, for example, in lubricants, functional fluids, ink, paints, coatings, nylons and resins. In addition, it can serve as a substrate for the synthesis of estolides, a novel lipid class with economic importance in the cosmetic, coating, and lubricant industries. The only commercial source of hydroxyl oil is *R. communis*, in which ricinoleic acid accumulates to up to 90% of the total fatty acids in seeds. However, castor bean has poor agronomic characteristics, rendering it unsuitable for modern agriculture practices. A potential

solution to this problem is to use a conventional breeding approach to develop an agronomically suitable variety. However, such an approach is time-consuming and labor-intensive, with uncertain results. A biotechnological approach, on the other hand, allows the introduction of specific genes into plants and has the potential to improve target traits in a short time frame. Thus, the genetic engineering of oilseed crops has been viewed as a cost-effective, time-saving alternative method of producing large volumes of ricinoleic acid. A desaturase-like fatty acid hydroxylase gene (*CpFAH*) involved in ricinoleic acid synthesis was recently cloned from the oleaginous fungus *Clavicep purpurea* (Meesapyodsuk & Qiu, 2008). A putative diacylglycerol acyltransferase (CpDGAT2) thought to be involved in the last step of TG assembly was also identified from the same species in our laboratory. This research focuses on the characterization of its enzymatic properties specifically, the ability of CpDGAT2 to synthesize ricinoleic acid-containing TGs and the substrate preference of the cloned enzyme. As well, the expression patterns of *CpFAH* and *CpDGAT2*, two key genes in the biosynthesis and assembly of ricinoleic acid, were evaluated.

2. LITERATURE REVIEW

2.1 Triacylglycerol biosynthesis.

All eukaryotic and some prokaryotic organisms have the ability to synthesize triacylglycerols, the most common energy reserve in animals, plants, and eukaryotic microorganisms. TGs represent a very effective energy source; for example, up to six-fold more energy can be stored in TGs than in the same amount of hydrated glycogen (Alvarez and Steinbuchel, 2002). In plants, TGs are mainly stored in seeds where they are the major component of seed oil. In animals, TGs are synthesized in various organs, with the liver and intestine being the most active, and are stored predominantly in adipose tissues. The major role of TGs is to store fatty acids, which can be used for energy production through β-oxidation (Athenstaedt and Daum, 2006). TG synthesis is also important for the normal physiology of cells, protecting cells from the potentially harmful effects of an excess of free fatty acids. In animals, TGs can serve as an insulator in adipose tissues and as a water barrier for skin. TG synthesis also results in the formation of lipid particles, which transport endogenously synthesized and exogenously supplied FAs to serve as building blocks for the production of membrane lipids (Voelker, 2000).

TG is a glyceride in which a glycerol is acylated with three fatty acids. There are two pathways for TG biosynthesis: the glycerol phosphate or Kennedy pathway (Kennedy, 1961) and the monoacylglycerol pathway. Both pathways can occur simultaneously in organisms that produce TGs; however, one may dominate depending on the cell or tissue type. For example, in mammals the monoacylglycerol pathway is the dominant pathway in the small intestine. Dietary TGs are hydrolyzed into free fatty

acids (FFA) and monoacylglycerol (MAG) in the lumen of the small intestine and are re-esterified into TGs inside the enterocytes of the intestine (Kayden et al., 1967). This TG re-biosynthesis is believed to take place in the endoplasmic reticulum. Newly synthesized TGs are released in the form of lipid particles or oil bodies (Weiss et al., 1960), in which neutral lipids form a matrix surrounded by a phospholipid monolayer embedded with various proteins (Tzen et al., 1993).

During the last few years a number of enzymes involved in TG biosynthesis have been characterized at the molecular level, with the majority being from the Kennedy pathway. The initiation of this pathway in yeast, plants and mammals starts with the synthesis of glycerol-3-phosphate, which is then acylated at the *sn-1* position by glycerol-3-phosphate acyltransferase to produce lysophosphatidate (Fig. 1.1). In mammalian and yeast cells, lysophosphatidate can also be generated by the action of dihydroxyacetone acyltransferase, which acylates a fatty acyl-CoA to dihydroxyacetone phosphate. The product is then reduced by a NADPH-dependant reaction, forming lysophosphatidate (Sorger and Daum, 2003). The second step in the Kennedy pathway, catalyzed by lysophosphatidate acyltransferase, is the acylation of lysophosphatidate at the *sn-2* position, producing phosphatidate. A phosphatidate phosphatase then releases the phosphate group from the *sn-3* position and generates diacylglycerol (DAG), which is used by diacylglycerol acyltransferase to produce TGs. Phosphatidate can also be generated by the action of phospholipase D which cleaves the functional group of various phospholipids, and phospholipase C can also convert phospholipids to DAGs. The final step in TG biosynthesis can proceed through either the acyl-CoA dependent or the acyl-CoA independent pathways. In the former pathway, a diacylglycerol

acyltransferase (DGAT) directly acylates DAG with a fatty acyl-CoA at the *sn-3* position, while in the latter pathway a phospholipid diacylglycerol acyltransferase uses phospholipids as the acyl-donor for the acylation of DAG at the sn-3 position. Additionally, a diacylglycerol transacylase has been shown to catalyze a transesterification reaction using two molecules of DAG to form TG and MAG (Lehner and Kuksis, 1993). However, no such enzyme has been purified or studied at the molecular level.

In the monoacylglycerol pathway of TG biosynthesis, hydrolysis of dietary lipids in the small intestine by pancreatic lipase results in the production of a mixture of 2-MAGs and FFAs, which are then reacylated into TG in the enterocyte cells of the intestine. In this TG-synthetic pathway (Fig. 1.2), 2-monoacyl-*sn*-glycerol is first acylated at the *sn-1* or *sn-3* position by monoacylglycerol acyltransferase (MGAT) resulting in 1,2 or 2,3-diacyl-*sn*-glycerol. The major product of MGAT is the 1,2-diacyl-*sn*-glycerol, with 2,3-diacyl-*sn*-glycerol being present only in small amounts. The last step in the monoacylglycerol pathway is catalyzed by diacylglycerol acyltransferase (DGAT) where DAG is acylated with the third fatty acid, giving rise to TG.

Despite the distinct pathways leading to TG synthesis, the final step in TG synthesis, which is catalyzed by a DGAT, is common to all these pathways. Three types of enzymes with DGAT activities have been identified. DGAT1 has high homology to acyl-CoA:cholesterol acyltransferases (ACAT) and indeed was first identified in mouse via homology searches (Cases et al., 1998). A second DGAT enzyme, namely DGAT2, sharing no homology with DGAT1, was first cloned from *Mortierella*

ramanniana by Lardizabal et al., (2001). In addition, Sandager et al., (2002) discovered that the *ARE1* and *ARE2* genes from yeast, coding for sterol acyltransferases, also show DGAT activity.

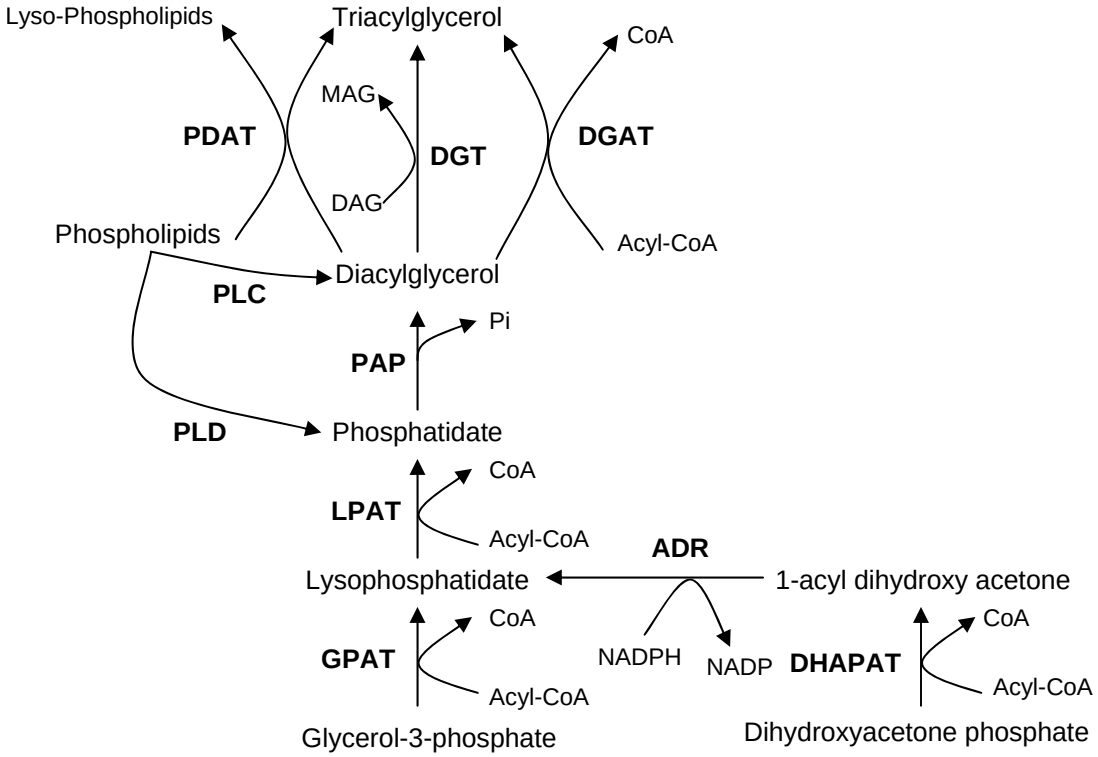


Figure 1.1 Kennedy pathway of TG biosynthesis. GPAT, glycerol-3-phospate acyltransferase; DAHPAT, dihydroxyacetone phosphate acyltranferase; ADR, 1-acyl dihydroxyacetone reductase; LPAT, lysophosphatidate acyltransferase; PAP, phasphatidate phosphatase; PLD, phospholipase D; PLC, phospholipase C; PDAT, phospholipids diacylglycerol acyltransferase; DGT, diacylglycerol transcyclase; DGAT, diacylglycerol acyltransferase.

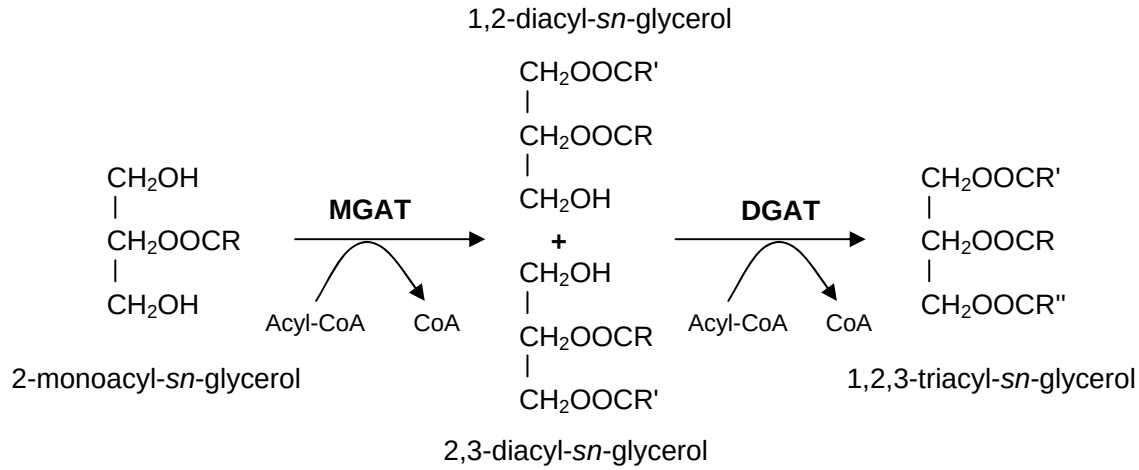


Figure 1.2 Monoacylglycerol pathway of TG biosynthesis. MGAT, monoacylglycerol acyltransferase; DGAT, diacylglycerol acyltransferase.

TG biosynthesis occurs mainly in the endoplasmic reticulum, but lipid particles are also able to synthesize TG. In yeast, lipid particles were found to possess acyltransferase activities responsible for the formation of phosphatidate. *Gat1*, which catalyzes the acylation of glycerol-3-phosphate and dihydroxyactone phosphate, *Ary1*, which acts as a reductase on 1-acyl dihydroxyacetone phosphate (Athenstaedt et al., 1999b), and *Slc1*, which has acyltransferase activity on lysophosphatidate (Nagiec et al., 1993) have been determined to be components of lipid particles (Athenstaedt and Daum, 1997, Athenstaedt et al., 1999a). Although phosphatidate phosphatase, which converts phosphatidate to diacylglycerol, is believed to be localized in the cytosol, it may have access to the surface of lipid particles or to the cytosolic part close to lipd particles. Dga1 (DGAT2), which catalyzes diacylglycerol acylation, is a lipid particle protein (Sorger and Daum, 2002). Mitochondria might also serve as a site for the

synthesis of TGs, as they contain genes homologous to *Gat1* and *Gat2*, which are responsible for the formation of lysophosphatidate (Athenstaedt et al., 1999a).

Although TG biosynthesis is important for normal growth and development, the excessive accumulation of TGs in adipose tissue in humans may result in obesity. Therefore DAGT1 and DGAT2, the key enzymes catalyzing TG formation, have recently drawn tremendous scientific attention. Studies on mice lacking both DGATs have associated the lack of enzymatic activity with a reduction in TG formation and a resistance to diet-induced obesity (Smith et al., 2000). This implies that inhibition of DGAT activity may have potential medical applications in the treatment of obesity.

2.2 Unusual Fatty acid Biosynthesis.

Fatty acid biosynthesis is essential for nearly all living organisms. The rate of fatty acid synthesis depends on the species and the cell type, and in certain cells and tissues where the demand for fatty acids is greater, higher levels of fatty acid synthesis occurs. An example of this is the plant oilseed, which can accumulate up to 60% of the seed weight as triacylglycerol.

Common fatty acids in vegetable oil are those of 16 or 18 carbons in chain length with one to three double bonds. However, some plants and microorganisms can produce high levels of unusual fatty acids that differ from common fatty acids in terms of chain length, or that possess functional groups such as hydroxyl, epoxy, or acetylenic groups (Shanklin and Cahoon, 1998). In many cases these unusual fatty acids have valuable chemical or physical properties with potential industrial uses. For example, erucic acid (22:1), a major constituent of hight erusic acid-*Brassica napus* seed oil, can

be readily converted to erucamide which is extensively used as a slip agent in plastic film (Ohlrogge, 1994). In addition, vernolic acid (18:1-9c-12,13epoxy), an epoxy fatty acid found in the seed oil of *Vernonia galamensis*, has a low viscosity and can be used as a non-volatile solvent in oil-based paints (Yu et al., 2006). Hydroxyl fatty acids are highly important industrial fatty acids and occur in a wide variety of organisms, with plants being the major producers. Characteristic of these fatty acids is the presence of hydroxyl groups at various positions of the carbon chain. Based on the number of hydroxy groups, hydroxy fatty acids can be classified into two types: fatty acids with a single hydroxy group and fatty acids with more than one hydroxy group. In the monohydroxy group, ω-hydroxy acids have the hydroxy group at the methyl end of the carbon chain. They are the major component of suberin in plant cell walls and cutin in plant cuticle (Pollard et al., 2008). Kamlolenic acid (18:3-9c, 11t, 13t-18OH), an ω-hydroxy fatty acid found in seeds of *Mallotus phillipinensis*, can be used in rapid-drying paints and varnishes and as a fixative in cosmetic preparations. In addition, ω-hydroxy acids are found in the cell wall of green algae (Blokker et al., 1998) and in the royal jelly of nurse bees (Noda et al., 2005).

Other monohydroxyl fatty acids include avenoleic acid (18:2-6c, 9c-15OH), an oat-specific hydroxyl fatty acid (Hamberg et al., 1998); lesquerolic (20:1-11c-14OH), densipolic (18:2-9c, 15c-12OH) and auricolic (20:2-11c, 17c-14OH) acids found in oil of *Lesquerella* species (Smith et al., 1962), β-dimorphecolic acid (18:2-10t, 12t-9OH), which is present in seed oil of *Dimorhotheca pluvialis* (Cuperus et al., 1996), and ricinoleic acid (18:1-9c-12OH), the major fatty acid in seeds of *Ricinus communis* and sclerotia of the fungus *Claviceps purpurea*. These monohydroxyl fatty acids,

particularly ricinoleic acid, have many industrial applications including the production of food additives, textiles, paper, plastics, perfumes and cosmetics, pharmaceuticals, paints, inks, adhesives, lubricants and Turkey-Red oil. Although most hydroxy fatty acids are found in plants, some species present are in animal wool waxes, skin lipids and brain tissues. Examples in animals include cerebronic (24:0-2OH) and hydroxynervonic (24:1-15c-2OH) acids, which are present in glycosphingolipids found in nervous tissue. Another group of monohydroxy fatty acids, found in bacteria, are hydroxy fatty acids with a hydroxy group at the third carbon. 3-hydroxy fatty acids are unique components of endotoxin, a lipopolysaccharide found in the outer-membrane of various Gram-negative bacteria. This fatty acid can be used as a biomarker for estimation of bacterial numbers (Lee et al., 2007). Additionally, 16 or 18 carbon 3-hydroxy fatty acids with two conjugated double bonds from the green macroalgae, (*Tydemania expeditionis*) are believed to have moderate antitumor activity (Jiang et al., 2008).

Polyhydroxy fatty acids have two or more hydroxyl groups attached to the carbon chain. Examples of these fatty acids are 13, 14-dihydroxydocosanoic acid (22:0-13, 14OH) and 11, 12-dihydroxyeicosanoic acid (20:0-11, 12OH) found in seed oil of *Brassicaceae* (Romanus et al., 2008). Tetrapedic (20:0-3,7OH) and byrsonic (22:0-3,7OH) acids have been isolated from floral oil of plants (Reis et al., 2007). Phaseolic acid (12:0, 2O- 5, 8, 12OH) was identified from pea, and showed elongation-stimulating properties in stem segments (Farmer, 1994).

Epoxy fatty acids are found in triacylglycerol fractions of seed oil as well as in cutin and suberin of plants. They are characterized by an 18-carbon length with an

oxirane group in the chain. One such epoxy FA, vernolic acid, was first discovered by Gunstone, (1954) in *Vernonia anthelmintica*, and can account for 72% of the total fatty acids in the seeds of this plant. Since then, many plant species such as *Euphorbia lagasceae* (Borch-Jensen and Mollerup, 1996), *Vernonia galamensis* and *Crepis aurea* (Badami and Patil, 1980) have also been found to produce this unusual fatty acid. As well, an isomeric form of vernolic acid, namely coronaric acid (18:1-12c-9,10epoxy) was identified in seed oil of *Chrysanthemum coronarium* (Morris and Wharry, 1966). Vernolic and coronaric acids are also commonly present in cutin and suberin where they represent up to 34% of the lipid content. Small quantities of epoxy-stearic acid (18:0-9,10epoxy) have been detected in peanut (*Arachis hypogeae*) (Hammond et al., 1997). In addition, a 20 carbon unsaturated epoxy acid, alchornic acid (20:1-11c-14,15O) was found in seed oil of *Alchornea cordifolia* (Kleiman et al., 1977). Among plants producing epoxy fatty acids, *Vernonia galamensis* L. is the most important in terms of the potential for industrial use. *V. galamensis* seed contains about 40 to 42% oil, of which 73 to 80% is vernolic acid. Products derived from vernonia oil include adhesives, varnishes and paints and industrial coatings. Vernonia oil is also used as a non-volatile solvent in oil-based paints due to its low viscosity, which allows easier incorporation of dry paint. Vernonia oil can also be used as a natural source of plasticizer and stabilizer for producing polyvinyl chloride, which is currently derived from petroleum.

Acetylenic acids are unusual fatty acids characterized by the presence of one or more triple bonds in the fatty acid chain. Of the many species of acetylenic acids known, only a few are derived from natural sources. Based on the number of triple

bonds present in the carbon chain, these natural acetylenic acids can be grouped into two categories, the monoacetylenic and polyacetylenic acids. The first monoacetylenic acid to be identified, tariric acid (18:1-6a), was described by a French chemist in the nineteenth century, and constitutes 95% of the total fatty acids in the seeds of *Picramnia tariri*. Stearolic (18:1-9a) and santalbic (20:2-9a, 11c) acids, two other monoacytelenic acids with distinctive biochemical features, were identified from seeds of *Exocarpus* and *Santalum*, respectively. Administration of these fatty acids to mice inhibited the activity of several lipoenzymes such as lipoxygenase and prosTGlandin synthetase, leading to changes in the fatty acid composition of adipose tissue, liver, and brain (Liu and Longmore, 1997). The monoacytelenic acid 6-octadecynoc acid (18:1-6a), isolated from the roots of *PanTGonia gigantifolia*, possesses anti-fungal properties, inhibiting the growth of fluconazole-susceptible and fuconazole-resistant *Candida ablicans* strains (Li et al., 2003). Certain acetylenic acids were also found to have anti-mycobacterial and anti-plasmodial activity, such as scleropyric acid (17:1-12a) from *Scleropyrum wallichianum* (Suksamrarn et al., 2005). In addition, acetylenic acids isolated from the marine sponge *Stellata* sp were shown to exhibit weak cytotoxicity against the human leukemia cell-line K562 (Lee et al., 2003). Recently, nine new monoacetylenic acids (gallicynoic acids) with 14 to 18 carbon atoms and one to three hydroxyl groups from the fungus *Coriolopsis gallica* were described (Zhou et al., 2008).

Polyacetylenic acids are fatty acids with two or more acetylenic groups. These fatty acids are generally unstable and susceptible to oxidation and pH-dependent decomposition, thus their industrial applications are limited. Polyacetylenic acids are

not commonly found in living organisms, but examples include the diynoic acid, 13,14-dihydrooropheic acid (18:4-9a, 11a-13t, 17t) from *Mitrephora celebica,* which has strong antimicrobial activity against *Staphylococcus aureus* and *Mycobacterium smegmatis* (Zgoda et al., 2001), and oropheic acid (18:4-9a, 11a, 13a-17t), a triynoic acid from the leaves of *Orophea enneadra* and *Mitrephora celebica* that exhibits significant anti-fungal activity, especially against the fungus *Cladosporium* (Zgoda et al., 2001).

Biosynthesis of unusual fatty acids starts with synthesis of common fatty acids, which are then processed by a variety of fatty acid-modification enzymes, resulting in chain elongation, desaturation or addition of a functional group. Fatty acid synthesis from C2 to C18 saturates in plants occurs in plastids through the activity of a group of enzymes called type II fatty acid synthase (FAS II) (Paul et al., 2001, Rawsthorne, 2002). The highly conserved FAS II system is also found in bacteria and is therefore referred as the prokaryotic type (Figure 2.1 and Table 2.1). In animals and yeasts, fatty acid biosynthesis occurs via the activity of a single enzyme with multi-functional domains, known as type I fatty acid synthase (FAS I) (Smith, 1994). Recent genome sequencing projects have revealed the existence of novel FAS II systems in fungi, parasites and plant mitochondria (Paul et al., 2001).

The initiation of fatty acid biosynthesis in plants and bacteria is a two step reaction catalyzed by acetyl-CoA carboxylase (ACC) (Albert and Vagelos, 1968), which produces malonyl-CoA through the cooperative action of three functional subunits (AccB, AccC and AccA-AccD). The first step is the energy dependent carboxylation of the biotin component of biotin carboxylase carrier protein (AccB)

catalyzed by biotin carboxylase (AccC) using bicarbonate as a substrate (1). The second step is the carboxyl transfer from AccB to acetyl-CoA catalyzed by carboxyltransferase (AccA- AccD), resulting in malonyl-CoA (2).

$$ATP + HCO_3^- + AccB \longleftrightarrow ADP + P_i + AccB\text{-}CO_2^- \quad (1)$$

$$AccB\text{-}CO_2^- + Acetyl\text{-}CoA \longleftrightarrow AccB + malonyl\text{-}CoA \quad (2)$$

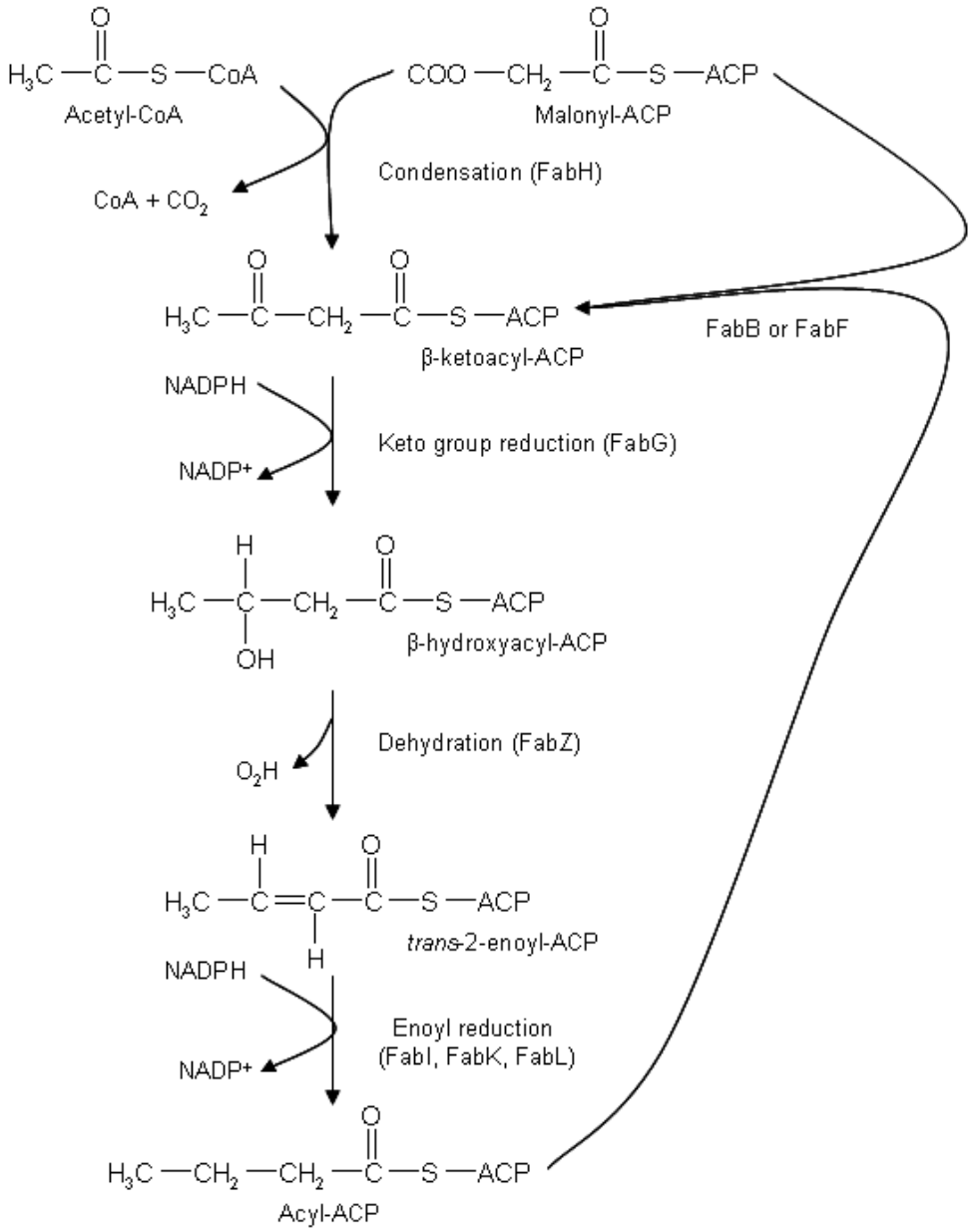
Table 2.1 Genes and enzymes of type II fatty acid synthase in *E. coli*.

Gene	Protein	Enzymatic activity
Acp	ACP	Acyl carrier protein
accA	AccA	Acetyl-CoA carboxylase, carboxyltransferase α-subunit
accB	AccB	Acetyl-CoA carboxylase, carboxybiotin carrier protein
accC	AccC	Acetyl-CoA carboxylase, biotin carboxylase
accD	AccD	Acetyl-CoA carboxylase, carboxytransferase β-subunit
fabD	FabD	Malonyl-CoA: ACP transacylase
fabH	FabH	β-ketoacyl-ACP synthase III
fabB	FabB	β-ketoacyl-ACP synthase I
fabF	FabF	β-ketoacyl-ACP synthase II
fabG	FabG	β-ketoacyl-ACP reductase
fabA	FabA	β-hydroxyacyl-ACP dehydratase/isomerase
fabZ	FabZ	β-hydroxyacyl-ACP dehydratase
fabI	FabI	*trans*-2-Enoyl-ACP reductase I
fabK	FabK	*trans*-2-Enoyl-ACP reductase II
fabL	FabL	*trans*-2-Enoyl-ACP reductase III

(Adopted from Heath et al., 2001).

During fatty acid synthesis, malonyl-CoA is transferred to acyl carrier protein (ACP) by a reaction catalyzed by malonyl-CoA: ACP transacylase (FabD) (Simon and

Slabas, 1998). ACP is a small acid protein required for the docking of substrates to enzymes (Zhang et al., 2001). Malonyl-ACP and acetyl-CoA then enter the first cycle of fatty acid synthesis.



(Adapted from Rock and Cronan, 1996)

Figure 2.1 Reaction sequences of type II fatty acid synthesis.

The entire fatty acid synthesis process contains four catalytic steps including condensation, keto group reduction, dehydration, and enoyl reduction. In the first step, malonyl-ACP is condensed with acetyl-CoA, resulting in β-ketobutyryl-ACP (Heath et al., 2001). This reaction is catalyzed by β-ketoacyl-ACP synthase III (KAS III) (FabH). Condensation of short-chain acyl-ACPs (from C_4 to C_{14}-acyl-ACP) is catalyzed by KAS I (FabB), while KAS II (FabF) only catalyzes the final condensation reaction using C_{16}-acyl-ACP as a substrate. Both KAS I and KAS II utilize malonyl-ACP and the end product of the fatty synthesis cycle for the condensation reaction (Rock and Cronan, 1996). In the first reductive step of the repetitive cycle of fatty acid synthesis, the keto group of the acyl chain is converted to a hydroxyl group in a NADPH-dependent reaction catalyzed by β-ketoacyl-ACP reductase (FabG) (Rock and Cronan, 1996). The resulting β-hydroxylacyl-ACP is further dehydrated to trans-2-acyl-ACP by β-hydroxylacyl-ACP dehydrase (FabA, FabZ), which removes a molecule of water from the acyl chain. The final reductive step is catalyzed by trans-2-enoyl-ACP reductase (FabI, FabK, FabL). In this NADPH-dependent reaction, the trans double bond is reduced, resulting in a saturated acyl-ACP. At least three isomers of trans-enoyl-ACP reductase have been identified in bacteria, but FabI appears to be the most highly conserved among prokaryotes (Baldock et al., 1998), while FabK and FabL are found only in a few species such as *Streptococci* and *Clostridia* (Heath et al., 2001).

Plant fatty acid synthesis occurs in plastids, and the acyl-ACPs generated in plastids are exported to the cytosol for further modification. During the export process, ACP is released by the action of acyl-ACP thioesterase or acyl-ACP hydrolase and the fatty acid is esterified to a CoA molecule by an acyl-CoA synthetase (Johnson et al.,

2002). In eukaryotes, including plants, fatty acid elongation takes place independently of FAS through a membrane bound elongase on the endoplasmic reticulum, with CoA acting as the acyl carrier molecule. Fatty acid elongation involves a condensation reaction using malonyl-CoA as the chain extending unit, followed by a β-keto group reduction, a dehydration reaction, and finally an enoyl reduction, as in the FAS system.

Newly synthesized fatty acids are directed to the cytosol for the synthesis of phospholipids, which serve as substrates for fatty acid desaturation or for unusual fatty acid synthesis. Biochemical characterization of ricinoleic acid biosynthesis using microsomal fractions revealed that oleic acid linked to membrane phospholipids acts as the substrate for the synthesis of the hydroxyl acid (Bafor et al., 1991, Smith et al., 1992). The first fatty acid hydroxylase identified (RcFAH12), which is capable of adding a hydroxyl group onto the twelfth carbon of oleic acid, was cloned by searching for FAD2 homologues in an EST database generated from developing castor seeds (van de Loo et al., 1995). When *R. communis* FAH12 was heterologously expressed in *Arabidopsis* (Broun and Somerville, 1997) and tobacco (van de Loo et al., 1995), ricinoleic acid was the major novel fatty acid produced in seeds. In addition, small amounts of lesquerolic, auricolic, and densipolic acids were detected. Since then, additional FAD2-like fatty acid hydroxylases have been identified from *Lesquerella lindheimeri* (Dauk et al., 2007), *Lesquerella fendleri* (Broun et al., 1998), *Dimorphotheca sinuata* (Cahoon and Kinney, 2004), and *Claviceps purpurea* (Meesapyodsuk and Qiu, 2008). Other FAD2-like enzymes involved in unusual fatty acid synthesis include the epoxygenase and acetylenase enzymes involved in the biosynthesis of vernolic and crepenynic acids, respectively. An epoxygenase gene was

first identified from *Crepis palaestina* by degenerate RT-PCR, and expression of this gene in *Arabidopsis* generated up to 15% of vernolic acid in seeds (Lee et al., 1998). The *C. palaestina* epoxygenase adds an epoxy group in the 12 carbon of linoleic acid in the presence of NADH. It is 374 amino acids in length and the amino acid sequence shows 58% identity with *Arabidopsis* FAD2 and 53% identity with RcFAH12. Recently, a similar FAD2-like epoxygenase was cloned from *Stokesia laevis* using degenerate primers and functionally characterized in *Arabidopsis* (Hatanaka et al., 2004). A new type of epoxygenase, also involved in the biosynthesis of vernolic acid, was isolated from *Euphorbia lagascae* in 2002 (Cahoon et al., 2002a). The *E. lagascae* epoxygenase resembled a cytochrome P450-like monooxygenase and expressing this gene in tobacco callus and somatic soybean embryos resulted in accumulation of vernolic acid (Cahoon et al., 2002a).

An acetylenase gene involved in the biosynthesis of fatty acids with triple bonds was isolated from *Crepis alpina* by degenerate RT-PCR in late the 1990s (Lee et al., 1998). It encoded a 371 amino acid protein with 56% amino acid identity to Arabidopsis FAD2. Expression of this gene in *Arabidopsis* resulted in the production of 25% of crepenynic acid in seeds. This acetylenase converts the double bond at the 12^{th} position of linoleic acid to a triple bond, giving crepelynic acid. Since then, acetylenase genes have also been isolated from *Petroselinum crispum*, *Sclerotinia slerotiorum* and *Helianthus annuus* (Cahoon et al., 2002b).

FAD2-analogous enzymes involved in the biosynthesis of conjugated fatty acids have also been identified. Two Δ12 conjugases, responsible for synthesis of α-eleostearic acid (18:3-9*cis*, 11*trans*, 13*trans*) and α-parinaric acid (18:4-9*cis*, 11*trans*,

13*trans*, 15*trans*) in *Morordica charantia* and *Impatiens balsamica*, were isolated and characterized in transgenic soybean (Cahoon et al., 1999). A Δ9 conjugase from Calendula *officinalis* for the synthesis of calendic acid (18:3-8*trans*, 10*trans*, 12*cis*) was independently identified by two laboratories simultaneously (Cahoon et al., 2001, Qiu et al., 2001). Additional FAD2-like conjugases involved in the synthesis of other types of conjugated fatty acids have been isolated recently from *Punica granatum* (Iwabuchi et al., 2003) and *Aleurites fordii* (Dyer et al., 2002).

2.3 Diacylglycerol acyltransferase

Diacylglycerol acyltransferase (DGAT) catalyzes the final step in TG biosynthesis, using diacylglycerol and fatty acyl-CoA as substrates. DGAT activity was first reported in 1956 (Weiss et al., 1960), but only in the last few decades have DGAT genes been cloned and characterized at the molecular level. There are at least two types of DGATs, namely type I DGAT (DGAT1) and type II DGAT (DGAT2), and while these enzymes catalyze the same reaction, they share no or little similarity at the protein level.

DGAT protein was partially purified in 1980 (Polokoff and Bell, 1980), but it was not until 1998 that Cases et al., (1998) isolated a cDNA from mouse corresponding to DGAT (MmDGAT) by using homology searches of expressed sequence TG (EST) databases with acyl-CoA:cholesterol acyltransferase (ACAT) as the query sequence. The identified DGAT shared 20% identity at the amino acid level with ACAT. The mouse DGAT and homologous sequences were then named type I DGAT or DGAT1. DGAT1 from mouse was functionally characterized by expression in insect cells, where

MmDGAT exhibited a 5-fold increase in TG content compared to the control. The *MmDGAT* gene was initially tested for its ACAT activity, but results from this experiment were negative. A wide range of substrates, including DAG, were then used for activity assays, and high DGAT activity was observed. Since the first DGAT1 was cloned from mouse, DGAT1 genes have been identified from many different eukaryotic organisms including yeast, fungi, plants, animals, and invertebrates. DGAT1 belongs to a large protein family, the membrane-bound O-acyltransferases (MBOAT) (Hofmann, 2000). A characteristic of this family is the presence of a long hydrophobic region containing a histidine, which is likely an active-site residue. The MBOAT family contains mainly acytransferase proteins that transfer fatty acids onto hydroxyl groups of various substrates. Additional members of the MBOAT protein family include ACAT1, ACAT2, protein-cystein N-palmitoyltransferase, Ghrelin O-acyltransferase, Hedgehog acyltransferase, and wax synthase (Hofmann, 2000).

Along with its DGAT function, DGAT1 also exhibits other enzymatic activities. An *in vitro* assay using mouse DGAT1 showed that the enzyme catalyzes the esterification of a fatty acyl-CoA with monoacylglycerol, generating diacylglycerols, and therefore acts as an monoacylglycerol acyltransferase (MGAT) (Yen et al., 2005). In addition, mice lacking DGAT1 (*Dgat1* $^{-/-}$) have reduced levels of wax esters in their fur (Chen et al., 2002). Enzymatic assays of homogenized cells expressing DGAT1 demonstrated a wax synthase activity, resulting in formation of wax monoesters and wax diesters. Furthermore, over-expression of DGAT1 in cells led to a 10 fold increase in wax synthase activity (Yen et al., 2005). DGAT1 was also demonstrated to act as an acyl-CoA:retinol acyltransferase (ARAT), catalyzing the synthesis of retinyl esters

from retinol and fatty acyl-CoA substrates. Over-expression of DGAT1 in COS-7 cells resulted in the accumulation of higher amounts of retinyl esters. In addition, *Dgat1* $^{-/-}$ mice had lower levels of retinyl esters and consequently lower levels of ARAT activity (Yen et al., 2005). However, mutant mice showed a only a moderate reduction in tissue TG content while plasma TG levels were maintained at normal values, suggesting a limited DGAT activity compared to that of DGAT2 (Smith et al., 2000).

The existence of a second DGAT (DGAT2) was initially suggested by the work of Routaboul et al., (1999), who created an *Arabidopsis* mutant (ABX45) with a frameshift mutation near the 5' end of *TG1* gene (DGAT1), leading to a complete change in amino acid sequence. However, although *TG1* translation was disabled, TGs were still detected. Due to the fact that *Arabidopsis* has a single copy of *TG1* (Hobbs et al., 1999), Routaboul et al., (1999) concluded that an additional DGAT enzyme was present. In agreement with this, Smith et al., (2000) reported that mice lacking DGAT1 had a high amount of tissue TG. Further experiments based on these observations led eventually to the isolation of two DGAT2 cDNAs from the fungus *Mortierella ramanniana* (Lardizabal et al., 2001) and consequently to identification of various DGAT2 genes from fungi, plants, and animals (Table 2.2). Although the isolated genes exhibited DGAT activity, they were unrelated to the previously identified DGAT (DGAT1), which belongs to MBOAT family and shares ~20% identity with ACAT. DGAT2 belongs to the DAGAT protein family, as designated by Cases et al., (2001). DAGAT is a seven-member gene family including monoacylglycerol acyltransferases (MGAT1, MGAT2, MGAT3) and wax monoester synthase.

Table 2.2 Accession numbers of various DGAT2

Organism	Accession Nr.	Organism	Accession Nr.
Homo sapiens	AAQ88896	*Zea mays*	ACG38122
Mus musculus	AAH43447	*Oryza sativa*	BAD33251
Rattus norvegicus	AAH89846	*Mortierela remanniana* (B)	AAk84180
Bos Taurus	AAT78344	*Mortierela ramanniana* (A)	AAK84179
Xenopus tropicalis	AAH64191	*Yarrowia lipolytica*	CAT16409
Danio rerio	AAH96927	*Talaromyces stipitalus*	EED21737
Pan troglodytes	XP 522113	*Penicillium marneffei*	XP 002146410
Canis lupus famillaris	XP 542303	*Cryptococcus neofermans*	XM 571235
Monodelphis domestica	XP 001364751	*Aspergillus clavatus*	XP 001273210
Equus cabalus	XP 001495352	*Aspergillus niger*	XP 001296146
Xenopus laevis	Q6PA23	*Aspergillus terreus*	XP 001211961
Macaca mulata	XP 001083431	*Aspergillus oryzae*	XP 001822244
Ornithorhynchus anatinus	XP 001512404	*Aspergillus fumigatus*	XP 749138
Gallus gallus	XP 426251	*Pyrenophora tritici-repentis*	XP 001939093
Vernicia fordii	ABC94474	*Laccaria bicolor*	XP 001875017
Ricinus communis	AAY16324	*Neurospora crassa*	CAE76475
Vitis vinifera	CHO68497	*Noesartorya fischeri*	XP 001261291
Medicago truncatula	ACJ84867	*Saccharomyces cerevisiae*	NP 014888
Arabidopsis thaliana	NP 566952	*Schizosaccharomyces pombe*	XP 001713160

The biochemical characterization of DGAT2 showed differences in activity between *in vivo* and *in vitro* analyses. Cells over-expressing DGAT2 accumulated high amounts of TG in *in vivo* studies. However, *in vitro* analysis of cells over-expressing DGAT2 resulted in considerably lower activity (Stone et al., 2004). Therefore, the optimization of assay conditions or the presence of an unknown cofactor may be

needed for *in vitro* analysis of DGAT2 activity. In comparison with DGAT1, DGAT2 is more active in lower concentrations of acyl-CoA (≤50 μM) and less active when less than 50 mM of magnesium is used (Cases et al., 2001). It is unclear whether DGAT2 enzymes generally have a common preference for a certain acyl-CoA. A substrate specificity assay of mammalian DGAT2 expressed in insect cells showed a preferential utilization of oleoyl-CoA followed by palmitoyl-CoA, while activity levels with linoleoy- and arachidonyl-CoA were similar (Cases et al., 2001). On the other hand, tung tree DGAT2 showed a preference towards α-linolenoyl-CoA followed by palmitoyl-, linoleoyl-, and oleoyl-CoA (Shockey et al., 2006). Additionally, enzymatic assays using solubilized and non-solubilized samples of a fungal DGAT2 from *M. ramanniana* showed no significant preference in acyl donor substrates between 18:1-CoA and 12:0-CoA. However, both acyl-CoAs were preferentially incorporated into TG when small and medium chain-containing DAGs were used as acyl acceptors (Lardizabal et al., 2001). Little data has been produced regarding the specificity of DGAT2 for unusual fatty acids. This may be due to the fact that DGAT2 exhibits lower activity in *in vitro* assays than DGAT1 and further optimization of assay conditions is needed. In addition, the nature and the physical properties of unusual fatty acids make them unstable at optimum incubation temperature (Shockey et al., 2006). However, Skockey et al., (2006) used *in vivo* studies to investigate the triacylglycerol preference of DGAT2 isolated from *V. fordii*, a species which contains oil rich in α-eleostearic acid. Expression of tung tree DGAT1 and DGAT2 in *Δdga1* and *Δlro1* mutant yeast cells fed with α-eleostearic acid demonstrated a five-fold increase in the accumulation of α-eleastearic acid-containing TGs in the DGAT2 compared to the DGAT1 sample.

In addition, DGAT2 showed preferential synthesis of TGs containing polyunsaturated fatty acids when compared to DGAT1, which favored the production of monounsaturated TGs. Research on the acyl acceptor specificity of *R. communis* DGAT genes was performed by Lin et al., (1998). They studied the incorporation of radiolabeled ricinoleate, oleate, and linoleate in TGs after incubation with castor microsomes. Analysis of TG fractions by HPLC showed that diricinoleoyl-oleoyl-glycerol accounted for 11.9% of the total radioactivity detected (including free fatty acids) followed by triricinolein (8.5%), diricinoleoyl-linoleoyl-glycerol (4.0%), and diricinoleoyl-linolenoyl-glycerol (0.8%). The first DGAT2 enzyme involved in the unusual fatty acid synthesis was cloned by Kroon et al., (2006) from developing seeds of castor bean. Enzymatic activity of castor DGAT2 (RcDGAT2) was proven using sn-1,2-diricinolein as an acyl acceptor and [1-^{14}C]ricinoleoyl-CoA as an acyl donor. Coexpression of RcDGAT2 with the RcFAH in Arabidopsis led to increased accumulation of ricinoleic acid (up to 30%) (Burgal et al., 2008). Analysis of the TAG fraction revealed that in the co-expression line triricinolein molecules were increased by up to 17 folds. An *in vitro* enzymatic assay of yeast expressing the RcDGAT2 showed a preference in utilization of DAGs containing ricinoleic acid as an acyl acceptor (Burgal et al., 2008). Overall, the data generated regarding DGAT2 substrate specificity indicates that DGAT2 enzymes do not have a common preference for a specific acyl chain donor or acyl acceptor. Instead, it appears that DGAT2 prefers to utilize acyl substrates that are characteristic for the species from which the gene was isolated. In addition, *in vivo* studies showed the DGAT2 may have higher specificity towards unusual fatty acids than DGAT1.

DGAT1 and DGAT2 are found mainly in the endoplasmic reticulum (Cases et al., 2001), but the specific locations of DGAT1 and DGAT2 appear to be somewhat different. Expression of the DGAT1 and DGAT2 from tung tree in a tobacco cell line showed that each enzyme is localized to distinct punctate areas of the endoplasmic reticulum, suggesting that the enzymes were in different endoplasmic reticulum subdomains (Shockey et al., 2006). DGAT2 is an integral membrane protein predicted to have only one or two transmembrane domains (Lardizabal et al., 2001) with the N and C termini oriented towards the cytosol (Shockey et al., 2006, Stone et al., 2006). The location of the active site of DGAT enzymes is controversial, with both the endoplasmic reticulum lumen and cytosolic sides being candidates (Waterman et al., 2002). However, according to Stone et al., (2006), site directed muTGenesis of the highly conserved amino acid sequence HPHG, located approximately 45 amino acids after the transmembrane domain, decreased DGAT activity by 50-60% compared to the wild type. Thus, this conserved domain, present in all DGAT2 enzymes, may have important catalytic activity and suggests the active site is oriented towards the cytosol. Prior to the work by Stone et al.,(2006), Abo-Hashema et al., (1999) suggested that DGAT activity was oriented towards the lumen, since newly synthesized TGs could be washed from fused microsomes with dibutyl phthalate, an effective TG removal agent that acts on the external site of microsomes. However, a latent luminal activity of DGAT was also suggested by Abo-Hashema et al., (1999). This latent activity was not present, according to topology analysis, in mouse DGAT2 but only in DGAT1 since microsomes of *Dgat1* $^{-/-}$ mice showed decreased both overt (cytosolic) and latent (luminal) activities (Stone et al., 2006). DGAT2 enzymes are about 50 amino acids

longer at the N termini than enzymes belong to MBOAT family, and this region probably includes domains that are responsible for substrate specificity. However, among DAGAT family members the most conserved domains are found in the C termini. In addition to the HPHG domain, DGAT2 contains the consensus sequence FLxLxxxn (where n is a nonpolar amino acid) within the transmembrane domain (amino acids 80 – 87 in murine DGAT2); this consensus sequence is thought to be a putative neutral lipid-binding site influencing DAG substrate selectivity (Stone et al., 2006). Site-directed muTGenesis of this domain resulted in a decrease of DGAT activity. Similar domains are also present in other proteins that bind to or metabolize neutral lipids, such as cholesterol ester transfer protein, hormone-sensitive lipase, lecithin:cholesterol acyltransferase, cholesterol 7α-hydrolase, cholesterol esterase, and triacylglycerol hydrolase (Alam et al., 2006).

DGAT2 catalyzes the committed step in biosynthesis of TG, which is the major type of neutral lipid and the major component of seed oil. TGs are considered a valuable resource for dietary consumption and industrial usage. However, excessive accumulation of TG in human tissues results in obesity, which ultimately leads to arteriosclerosis, cardiovascular diseases and organ dysfunction. Therefore, understanding DGAT2 function at the molecular and biochemical levels is crucial for potentially controlling TG synthesis in humans, as well as for development of economically important oil-producing crops.

2.4 Claviceps purpurea

The genus *Claviceps* includes parasitic fungi which form specialized structures called sclerotia on grasses. Claviceps species belong into family Clavicipitaceae, order Hypocreales. Initially, the morphology of the conidiogenous stromata led some mycologists to classify Clavicipitaceae in the order Xylariales or to erect the order Clavicipitles (Gaumann, 1952). However, molecular studies in taxonomical relationship among members of Hypocreales and the family Clavicipitaceae revealed that Clavicipitaceae belongs in the order Hypocreales (Kuldau et al., 1997). The geographical distribution of *Claviceps* includes a wide range of regions such as: American tropics and subtropics, African and South Asian subtropical forests, Australia, and Europe. According to Langdon's (1954) hypothesis based on biogeography and the type of sclerotia, *Claviceps* is of tropical origin and the species with advanced sclerotia resistant to xeric conditions and temperature variations may have evolved as their hosts spread from the tropical forests to less favorable areas. Members of the genus *Claviceps*, also called ergot fungi, parasitize more than 600 species of plants including rye, wheat, barley, rice, corn, millet and oat (Tudzynski and Scheffer, 2004). The genus includes more than 30 species with the most representative and studied being *Claviceps purpurea*.

C. purpurea infects more than 400 species causing economic damage in cereal crops not because of reduced yields but due to contamination of the harvested grains. The fungus produces ergot sclerotia, which are specialized structures containing peptide alkaloids that when ingested can have deleterious effects on the central nervous system of mammals. During the Middle Ages, epidemic outbreaks of *C. purpurea* in rye

caused ergotoxicosis/ergotism, referred to as “St Anthony’s fire”, resulting in death or mutilation of thousands of victims. However, the alkaloid content of the sclerotia is of pharmaceutical importance, including the production of the hallucinogen lysergic acid diethylamide (LSD), and therefore has been widely investigated (Tudzynski et al., 2001). *C. purpurea* infects exclusively young and usually unfertilized ovaries with spores that follow nearly the same path as the pollen takes for fertilization, of the inflorescences of grasses, sedges, and rushes. During infection, the ovary is replaced by a specialized fungal structure called a sclerotium, which is a hard compact mass of fungal tissues that is generally four times bigger than the host seed. The disease caused by *Claviceps* and the sclerotia have the common name ergot, which is a general term that applies to all species of *Claviceps*.

Sclerotium is the sTGe of the fungus that survives through the winter. In order for the sclerotium to germinate, it requires one or two months of temperatures that varies from zero to 10 ^{0}C. This process is often referred to as “vernalization”. In the spring prior to flowering of grasses the sclerotium germinates, forming stromata which end with a capitulum. Inside the capitulum asci generate ascospores and the sexual cycle of *C. purpurea* begins. Moisture or rainfall is required for ascospores to be released. The spores are ejected into the air and are carried by air currents to grass flowers where they affect only the ovaries of the flowers. Susceptibility of infection varies among grasses. Flowers are more susceptible from the beginning of flowering until fertilization. Within a week after infection conidia are produced which represent the asexual cycle of the fungus. Conidia generate conidiospores which are mixed with the plant sap forming a sticky substance called honeydew. Secondary spread can occur by movement of

conidiospores to healthy plants by direct contact or by insect pollination. In the early sTGes of infection, honeydew is accumulated and dripped from the infected flowers. This sugar-like substance attracts flies and other insects which help in the propagation of the disease in non-infected plants. Many cycles of infections followed by transfer of conidia can occur as long as there are unpollinated/unfertilized flowers available. Eventually the number of susceptible plants decrease, or the crops are harvested, and *Claviceps purpurea* must overcome a significant period of time without a host. Within two weeks after infection sclerotia begin to appear which usually coincides with maturity of infected grasses. The sclerotium provides the ideal biological form for the transactional period under environmental stress. Once favorable conditions reappear at the beginning of the next crop season the sclerotium germinate.

Sclerotium size and shape is dependent on the space available inside the floral cavity. For example, sclerotia of *C. purpurea* produced in *Poa annua* are about 1-2mm long while the sclerotia of the same specie produced in *Secale cereale* are up to 50mm (Pazoutova, 2002). The function of sclerotia is that of a dormant or resting structure. *C. purpurea* has the most organized sclerotium structure within Clavicipitaceae. It is composed of thick-walled cells which contain lipids that are used as an energy source during the dormant period, and provides the necessary energy for germination. The cortex of the sclerotium is formed by thick layers of dead cells making it resistant to low temperatures and impermeable to other organisms (Tsukiboshi, et al., 1999). *C. purpurea* sclerotium is dark colored, classified as a type III elongated sclerotium and is ovoid to cylindrical in shape (Langdon, 1954). On the distal tip of the sclerotium is

usually a cap formed from the remnants of the sphacelial tissues. This type of sclerotium is found in all gramineous species.

The glycerides present in the sclerotium of *C. purpurea* account for up to 30% of dry weight and contain 24 – 35% ricinoleic acid (Morris and Hall, 1966). However, ricinoleic acid in sclerotium produced in submerged cultures, in some instances, can reach values as high as 50% of the total fatty acids (Kren et al., 1985). Ergot oil is mainly composed of acylglycerols, comprising 60% of the total lipids and therefore are the major class in sclerotia oil (Batrakov and Tolkachev, 1997). It has long been found the acylglycerols extracted from *C. purpurea* contain ricinoleic acid which is further acylated at the hydroxyl group (Bharucha and Gunstone, 1957). Studies performed by Morris and Hall, (1966) revealed that ergot oil contains numerous estolide types of acylglycerols, including mono-, di-, and tri-estolides triacylglycerols (TGs), which are composed of more than three fatty acids (Fig. 2.2). Furthermore, Batrakov and Tolkachev, (1997) identified six types of estolides TGs with mono- and di-estolides being 86% of the total TG fractions. Additional types were estolides with six fatty acids (4.8%), seven fatty acids (1.1%), eight fatty acids (0.3%), and ten fatty acids (0.2%).

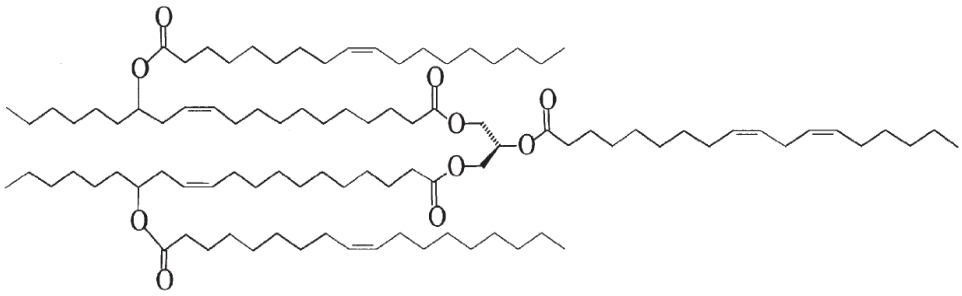


Figure 2.2 Triacylglycerol estolide

Estolides is a generic name for linear oligomeric polyesters of hydroxyl fatty acids in which the carboxyl group and hydroxyl group of hydroxyl fatty acids are dehydrated to form oligomers (Achaya, 1971). Estolides can be formed by a normal or a hydroxyl fatty acid esterifying a hydroxyl fatty acid. These are mainly found in some specialized TGs which have hydroxyl fatty acids in the *sn*-3, *sn*-2, or *sn*-1 position acylated with other fatty acids, forming tetra-, penta-, or hexaester glycerides, with *C. purpurea* being the only known natural source. Estolides made from ricinoleic acid are used in various industrial fields. These new functional fluids have a rapidly growing application in cosmetics, coating, and biodegradable lubricants. In skin care systems, they provide significant moisturization benefits. In addition, they improve intra-fiber moisture retention, restore elasticity, and prevent mechanical damage. To date several ways for *in vitro* synthesis of ricinoleic acid estolides have been found, but with limited success for commercialization of those estolides. Chemically synthesized estolides present problems in coloration and odor due to high reaction temperature needed during the synthetic process. Enzymatic biosynthesis of ricinoleic acid estolides by the catalytic action of lipase requires a very stable system with respect to temperature, pressure, pH, and water concentration, making the production of estolides vulnerable to mechanical damage thus raising their cost of production (Bodalo et al., 2005).

3. GENETIC ANALYSIS FOR SPECIATION OF CpDGAT2 DONOR WITHIN *CLAVICEPS PURPUREA*

3.1 Introduction

Geographic location and environmental conditions contribute to fungal diversity and distribution in plant ecosystems. They also have a major impact in the formation of biological characteristics such as morphology and reproduction. Therefore, these elements are widely used for the classification of fungi into orders and families as well as species identification. However, due to limitation of these characteristics and their sensitivity in environmental conditions, cultured-based methods provide little information regarding the relationship between species. In recent years phylogenetic approaches based on DNA molecular markers have become increasingly popular for species identification and species placement within genera (Taylor et al., 2000). Furthermore, phylogenetic analysis on molecular markers has been widely used for the identification of many fungi in which sexual reproduction is not known to occur. It has also been used for the identification of cryptic species in various fungal genera such as *Fusarium* (O'Donnell et al., 2000), *Coccidioides* (Koufopanou et al., 2001), and *Neurospora* (Dettmen et al., 2003).

Species delimitation based on host range and habitat specialization was common for *C. purpurea* taxonomy. Previous work done by Pazoutova et al., (2000) based on morphology and alkaloid chemistry led to the identification of three distinct groups, namely G1, G2, and G3 within *C. purpurea*. Recent phylogenetic and population genetic analysis performed by Douhan et al., (2008) using beta-tubulin, RAS-like

protein and internal transcribed spacer (ITS) as molecular tools, effectively analyzed the *C. purpurea* species delimitation within the three groups at the molecular level. In addition, Stevens et al (1997) used internal transcribed spacer (ITS) as a molecular tool for the speciation of *Pyrenophora* species within the genera. These data suggests that analysis of ITS rDNA sequence is a reliable method for species identification and species placement within a genera.

3.2 Objectives and Hypothesis

The goal of this study was the identification of a *C. purpurea* strain, from which *CpDGAT2* was previously isolated. In order to identify the *CpDGAT2* donor, analysis of ITS rDNA sequence was used in an alignment program with other ITS regions of various *C. purpurea* species.

The first hypothesis was that the ITS sequence of the strain would be available in GenBank database since various strains of *C. purpurea* have been widely investigated and molecularly analyzed based on ITS rDNA sequences. The second hypothesis was that the ITS region will be useful for placement of CpDGAT2 donor into one of the three distinct groups of *C. purpurea* by the ITS cluster analysis.

3.3 Materials and Methods

3.3.1 DNA extraction and PCR amplification of ITS rDNA sequence from *C. purpurea*

Population genetic analysis of the *C. purpurea* strain isolated in Manitoba and kindly provided by Dr. Yu Chen was performed using DNA sequences from the

internal transcribed spacer (ITS). Mycelium tissues for DNA extraction were grounded in liquid N_2 with a mortal and pestle. Genomic DNA was isolated using the UltraClean microbial DNA isolation kit (MoBio, Inc. CA) according to the manufacturer's instructions. The PCR for the amplification of the ITS was carried out in a total volume of 25 µl consisted of 2.5 µl of 10 x PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.5 µl of a 10 µM solution of each primer, 0.25 µl of Taq polymerase, 3.0 µl of DNA extract, and 17.5 µl of H_2O. The PCR amplification program consisted of an initial denaturation at 94 ^{0}C for 3 min followed by 30 cycles of 94 ^{0}C for 30 sec, 55 ^{0}C for 30 sec, and 72 ^{0}C for 1 min, and a final extension at 72 ^{0}C for 10 min. Primers used in this reaction were ITS1-F (5'CTTGGTCATTTGAGGAAGTAA) (Gardes and Bruns, 1993), which targets the 18S conserved sequence, as the forward primer and ITS4 (5'TCCTCCGCTTATTGATATGC) (White et al., 1990), which targets the 26S conserved sequence, as the reverse primer. The amplified fragment was then sent to PBI, National Research Council of Canada, Saskatoon, SK for sequencing.

3.3.2 Cluster analysis of *C. purpurea* gene donor ITS with various *C. purpurea* ITS sequences

An initial search for sequence similarity of the ITS fragment from *C. purpurea* (CpITS) was performed using a BLAST search of the GenBank nucleic acid database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was performed using the Geneious (version 4.0.2) software program. The Clustal X program (Chenna et al., 2003) was used to align CpITS with 18 ITS rDNA sequences from different isolates of *C. purpurea* belonging to the ecotypes G1, G2, and G3 (Douhan et al., 2008). Fungal

isolates, host plants and the location where isolates were recovered are listed in Table 3.1. The alignment file was converted into a cladogram using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) and the genetic distance model Jukes – Cantor and the obtained tree was similar to one created by Douhan et al., (2008).

Table 3.1 *Claviceps purpurea* isolates used for the ITS analysis.

Isolate	Group	Location	Host	Accession Nr.
165	G1	Zubri, Czech Republic	*Poa pratensis*	EU558999
204	G1	Lauderdale, Alabama	*Festuca arubinacea*	EU559000
428	G1	Hohenheim, Germany	*Secale cereale*	EU559002
NGE1	G1	Newfoundland, Canada	*Leymus mollis*	EU559010
WFA	G1	Nahcotta, Washington	*Festuca arubinacea*	EU559017
WLS	G1	Nahcotta, Washington	*Lolium* spp.	EU559019
236	G2	Vlei Pole u Bousova, Czech Republic	*Molinia coerulea*	EU559001
434	G2	Phillipsreuth, Germany	*Dactylis* spp.	EU559003
WCN2	G2	Willapa Bay, Washington	*Calamogrostis nutkaensis*	EU559013
WDG	G2	Leadbetter State Park, Washington	*Dactylis glomerata*	EU559014
WDS	G2	Willapa River, Washihgton	*Deschampsia caespitosa*	EU559016
WHS	G2	Long Beach, Washington	*Holcus lanatus*	EU559018
ARG1	G3	Argentina Celpa Marsh, Argentina	*Spartina densiflora*	EU559004
CDE1	G3	Point Reyes, California	*Spartina foliosa*	EU559005
CPE10	G3	Palo Alto, California	*Spartina foliosa*	EU559007
FSA1	G3	St. Augustine, Florida	*Spartina alterniflora*	EU559008
IRE12	G3	Dublin, Ireland	*Spartina anglica*	EU559009
RH2	G3	Rhode Island	*Spartina alterniflora*	EU559011

(Adapted from Douhan et al., 2008)

3.4 Results

PCR amplification using DNA isolated from the CpDGAT2 donor strain resulted in generation of a 579 nucleotide fragment. A BLAST search of the NCBI databases

indicated that this sequence (named CpITS) shared 99% nucleotide identity with the ITS sequences of four *C. purpurea* isolates (accession numbers: EU 559002.1, AB 160991.1, AB 162147.1 and AJ 309368.1). CpITS exhibited a lower level of homology with ITS rDNA regions from other *Claviceps* species and from unculturable or as-yet-unidentified fungal isolates. As shown in Figure 3.1., cluster analysis of the CpITS ITS sequence with other 18 ITS sequences belonging to the three different ecotypes placed CpITS into the G1 group, from which it differs only by six nucleotides. The G1 group differs from that G2 and G3 groups by 11 and nine base pairs, respectively (Douhan et al., 2008).

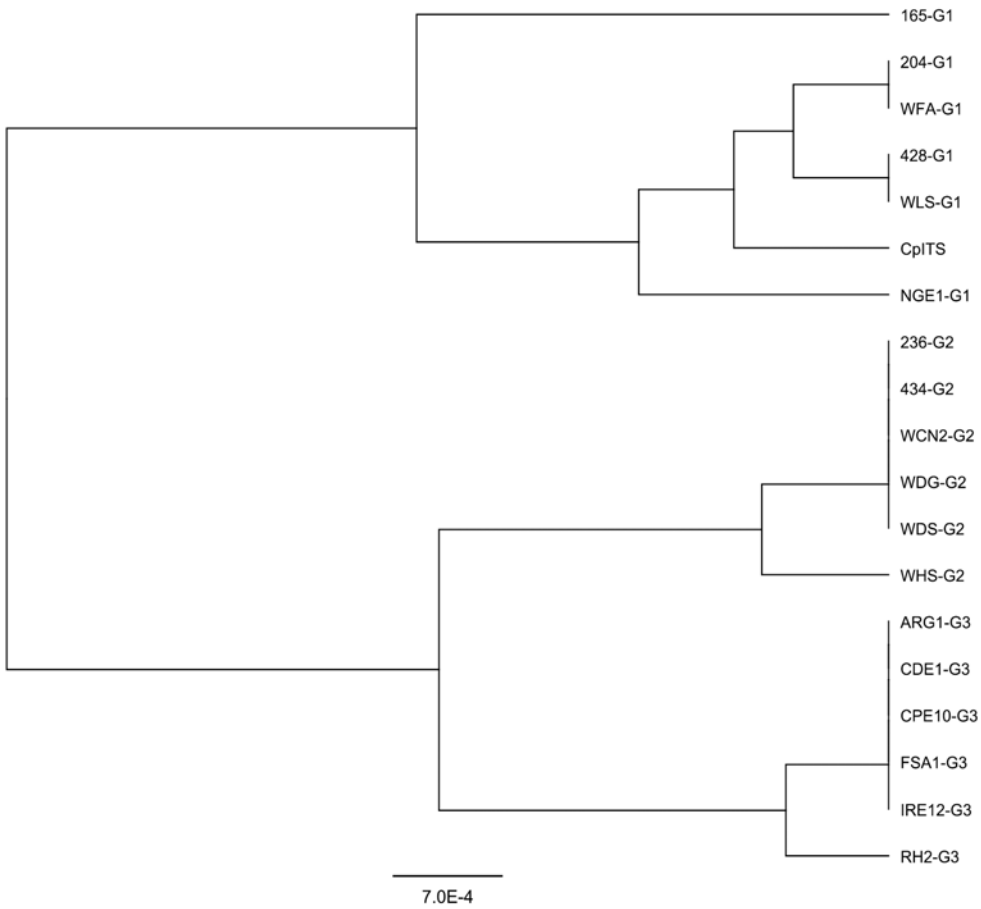


Figure 3.1 Cluster analysis of ITS rDNA sequences of *Claviceps purpurea*.

3.5 Discussion

The taxonomical classification of the *C. purpurea* strain from which the putative DGAT2 was isolated was performed by PCR amplification of the internal transcribed spacer (ITS) rDNA region and cluster analysis of ITS regions from 18 *C. purpurea* strains. Previous research classified *C. purpurea* strains into three groups within the species (Pazoutova et al., 2000). Each of these groups exhibit specific morphological characteristics and are associated with different habitats. For example, group G1 is the largest group that is associated with land grasses and has sclerotia that sink in the water. Group G2 is associated with grasses in "wet and shady" environments, while group G3 is associated with grasses in salt marsh habitats (Douhan et al., 2008). Groups G2 and G3 both possess the important difference in that their sclerotia can float on water (Fisher et al., 2005).

This study showed variance of ITS sequences among different strains of *C. purpurea* including our CpDGAT2 gene donor strain. The G1 group was significantly divergent from the G2 and G3 groups, while the G2 and G3 types were more closely related to one to another. Although G2 and G3 group show more similarity to each other than group G1, utilization of ITS rDNA sequences could still effectively separate them into the two distinct groups. The fact that G2 is more similar to G3 than to G1 was reinforced by the observation that G2 and G3 sclerocia float on the water while that of G1 sinks (Fisher et al., 2005). This classification is in agreement with previous studies using Random Amplification of Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) (Pazoutova et al., 2000) as well as phylogenetic and population analysis of beta-globulin, RAS-like protein, and ITS region of *C. purpurea*

(Douhan et al., 2008). Duncan et al., (2002) named G3 members as *C. purpurea* var. *spartinea* based on a combination of observations on host identity, sclerotia ecology and conidial morphology, although G3 isolates were found to be able to infect non-*Spartina* hosts in nature. Based on its conidial morphology (approximately 5.8 by 3.0 μm), the CpDGAT2 donor strain likely belongs to *C. purpurea* var. *purpurea* or *C. purpurea* f. sp. *secalis* (Pazoutova et al., 2000).

4. FUNCTIONAL EXPRESSION OF CpDGAT2 IN *SACCHAROMYCES CEREVISIAE* H1246.

4.1 Introduction

Storage lipids such as TGs naturally occur in plants, animals, fungi, yeast, and in bacteria. The biosynthesis of TGs has recently attracted a high level of the scientific interest due to their biological importance. The yeast *Sacchraromyces cerevisiae* accumulates TGs in lipid particles, which can account for up to 70% of total lipid content depending on the culture condition, while the total lipid content can vary between 3.5 to 10.7% of the cellular dry weight (Kalscheuer et al., 2004). The major TG-synthesizing enzyme is diacylglycerol acyltransferase (DGAT2) encoded by *DGA1*. In addition, phospholipid:diacylglycerol acytransferase encoded by *LRO1* is also involved in the synthesis of TGs in an acyl-independent reaction by using the *sn*-2 acyl group of phosphatidylcholine as the acyl donor. Furthermore, Sandager et al., (2001) demonstrated that acyl-coenzyme A:sterol acyltransferases encoded by *ARE1* and *ARE2* also exhibit a low level of DGAT activity.

S. cerevisiae, like *E. coli* for prokaryotes, is the most intensively studied model eukaryote in molecular and cell biology. It possesses many beneficial characteristics such as small size, short generation time and easy accessibility to genetic manipulation that render this organism an important scientific tool for the investigation of lipid metabolism. Enzymes involved in TG biosynthesis in yeast have been extensively studied and their mutants have been well characterized. The *S. cerevisiae* quadruple mutant H1246, in which all four genes (*DGA1, LRO1, ARE1 and ARE2*) involved in TG biosynthesis were disrupted, was generated by Sandager et al., (2001). Because it cannot synthesize TGs, it is a good host system for identification and characterization of TG-synthetic enzymes and thus, was chosen for biochemical characterization of *CpDGAT2*, a gene that codes for a putative diacylglycerol acyltransferase isolated previously by our laboratory.

4.2 Objectives and Hypothesis

The goal of this study was to determine whether the cloned *CpDGAT2* possesses diacylglycerol acyltransferase activity and if does, what kind of substrates it prefers in use for synthesizing TGs. This was accomplished by functional analysis of CpDGAT2 in the *S. cerevisiae* quadruple mutant H1246.

Our hypothesis is that the putative *CpDGAT2* clone encodes an acyltransferase protein with DGAT activity. We also hypothesized that CpDGAT2 could acylate ricinoleic acid to the *sn-3* position of DAG resulting in hydroxyl-TGs.

4.3 Materials and Methods

4.3.1. CpDGAT2 subcloning

The quadruple mutant *Saccharomyces cerevisiae* H1246 (Sandager et al., 2001) was used as an expression system for the putative CpDGAT2. The open reading frame of CpDGAT2 was PCR amplified with BamHI and NheI restriction sites incorporated into the 5' and 3' positions, respectively. Primers 5' CAGGATCCGAGATGGCAGCCGTCCAAGTC 3' (forward) and 5' ACGCTGCTCAAGACAAGATCTGCAGCTC 3' (reverse) were used to amplify a 1314 base pair fragment. PCR amplification of CpDGAT2 was conducted in a total reaction volume of 25 µl containing 0.5 µl of 10 µM of each primer, 3.0 µl of cDNA, 0.75 µl of 50 mM $MgCl_2$, 2.5 µl of 10X *Pfu* Ultra buffer, 0.5 µl of 10 mM dNTPs, and 0.25 µl of *Pfu* Ultra Taq polymerase, with the remaining volume being sterile distilled water. The PCR cycling conditions were 5 min at 95 ^{0}C followed by 30 cycles of 20 sec at 95 ^{0}C, 1 min at 54 ^{0}C, and 1.3 min at 72 ^{0}C, with a final extension of 5 min at 72 ^{0}C. The amplified product was analyzed by electrophoresis on a 1.0% agarose gel in 1X TAE buffer with 5 µg/ml ethidium bromide and purified using the QIAquick Gel Purification Kit (Qiagen, Mississauga) as per the manufacturer's instructions. The purified fragment was cloned into a pYES2.1/V5-His-TOPO vector (Invitrogen Canada Inc., Burlington, Ontario) according to the manufacturer's instructions. The resulting vector was then transformed into TOPO 10F' *E. coli* cells (Invitrogen Canada Inc., Burlington, Ontario). Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen, Mississauga) and sent to PBI, National Research Council of Canada, Saskatoon, SK for

sequencing. The pYES2.1/CpDGAT2 construct was then transformed into competent *S. cerevisiae* H1246 cells.

4.3.2 *S. cerevisiae* H1246 transformation

For the production of competent cells, H1246 yeast cells were streaked on YDP agar plates, which were then grown at 28 ^{0}C for two days. A single colony was used to inoculate 5 ml of YPD and the culture was grown overnight at 28 ^{0}C. Overnight cultures were then added to 100 ml of YPD pre-warmed to 28 ^{0}C and grown for 4 h at 28 ^{0}C with vigorous shaking (256 rpm). Cells were centrifuged at 3500 rpm for 5 min, resuspended in 50 ml of H_2O, and centrifuged at 3500 rpm for 5 min. The resulting cell pellet was resuspended in 1 ml of 100 mM Lithium acetate (Li-Acetate). Cells were then transferred to microcentrifuge tubes and centrifuged at the top speed for 15 sec in a benchtop centrifuge, the supernatant was discarded, and pellets were resuspended in 500 µl of 100 mM Li-acetate. Yeast transformation was performed according to Gietz and Woods, (2002). Briefly, 100 µl of cells were centrifuged at top speed in a benchtop for 15 sec. After the supernatant was removed, 357 µl of transformation mixture containing 240 µl of 50X PEG, 36 µl of 1M Li-Acetate, 50 µl of salmon sperm carrier DNA (2 mg/ml), and 31 µl of H_2O was added. Then 3 µl of pYES2.1/CpDGAT2 was added and the mixture was vigorously vortexed until the cell pellet was completely resuspended. The transformation mixture was then incubated at 30 ^{0}C for 30 min followed by a heat shock at 42 ^{0}C for 30 min. Cells were then centrifuged at 7000 rpm in a benchtop centrifuge and resuspended in 500 µl of H_2O. Selective plates (DOB minus uracil) were then spread with 100 µl of the mixture. For use as a control, *S.*

cerevisiae H1246 was also transformed with an empty pYES2.1 vector using the same protocol.

4.3.3 CpDGAT2 induction

For CpDGAT2 induction, the culture conditions were as described by Zou et al., (1999) with minor modifications. Briefly, single transformed colonies were cultured for two days in 20 ml DOB minus uracil medium at 28 ^{0}C on a rotary shaker (223 rpm). Cultures were then centrifuged at 4000 rpm for 4 min. The resulted cell pellet was washed once with water, centrifuged, washed once with inducing medium (DOB minus uracil + 2% galactose) and centrifuged again. Cells were then suspended in 20 ml DOB minus uracil and 2% galactose. The cell culture was grown at 28 ^{0}C in a rotary shaker (223 rpm) for another two days. After CpDGAT2 induction in galactose, cells were harvested by centrifugation at 4000 rpm for 4 min and washed twice with water. An additional feeding experiment with exogenously administrated ricinoleic acid was also performed. In this experiment, 250 μM of ricinoleic acid was added to the induction medium along with 0.1% of the detergent Tergitol. Wild type yeast (INVSc1) transformed with an empty pYES2.1 vector was used as a control. The conditions for cultures containing ricinoleic acid were the same as described above with the exception that 10 ml of growing and induction medium were used and 1% Tergitol was also included in the final cell wash (Meedapyodsuk et al., 2007).

4.3.4 Neutral lipid analysis

The TG-synthetic activity of CpDGAT2 was assessed with thin-layer chromatography (TLC). Total lipids were extracted from *S. cerevisiae* H1246 pYES2.1/CpDGAT2 and pYES2.1/BLANK constructs with chloroform:methanol 2:1 (v/v) according to Pillai et al., (1998) using a homogenizer and spotted onto preheated at 80 ^{0}C for two hours silica gel-G plates and developed in hexane:diethyl ether:acetic acid (70:30:1) (v/v/v) (Aitzetmuller et al., 1992). For the ricinoleic acid fed cultures total lipids were separated using a mobile phase of hexane:diethyl ether:acetic acid 70:140:3 (v/v/v) as described by Donaldson, (1977). For neutral lipid band visualization, the developed TLC plates were air-dried and sprayed with the lipid staining solution primuline (5mg primulin in acetone/water 80:20 v/v). Lipids were then observed under a transluminator.

4.4 Results

4.4.1 Molecular analysis of CpDGAT2

The CpDGAT2 cDNA was cloned by degenerate PT-PCR and RACE (rapid amplification of cDNA ends). It encodes a polypeptide with the molecular weight of 48.9 kDa and an isolectric point of 9.27. Phylogenetic analysis of CpDGAT2 and related proteins (Fig. 4.0) indicated that CpDGAT2 is tightly clustered with fungal and yeast DGAT2s, which are distantly related to DGAT2s from plants and animals.

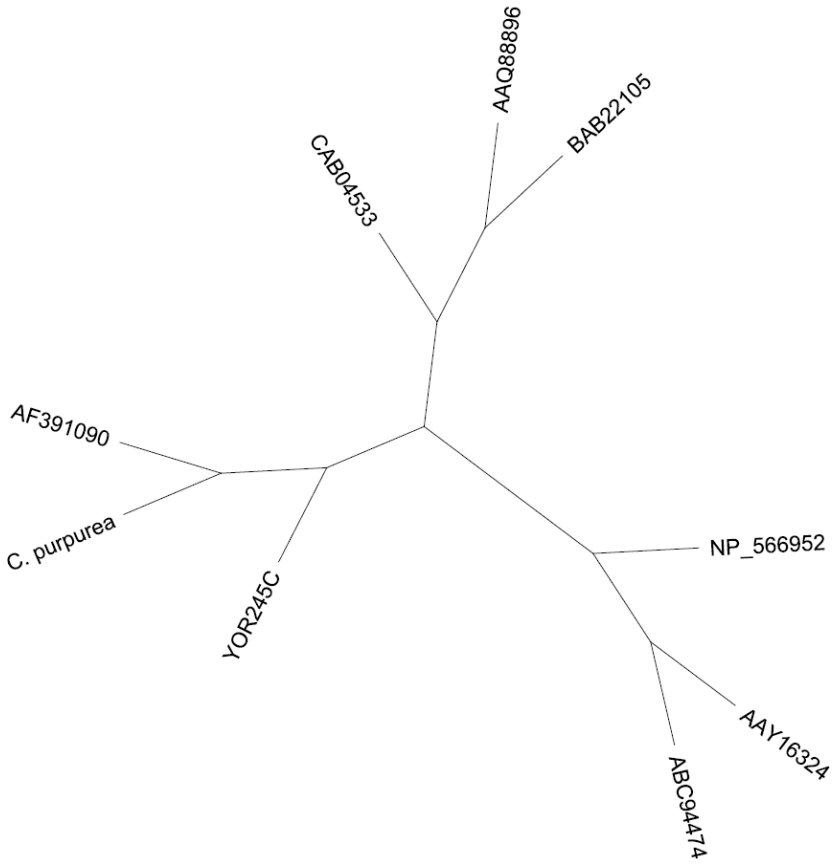


Figure 4.1 Unrooted tree of DGAT2 proteins. *Mortierela ramanniana* (AF391090), *Saccharomyces cerevisiae* (YOR245C), *Vernicia fordii* (ABC94474), *Ricinus communis* (AAY16324), *Arabidopsis thaliana* (NP_566952), *Mus musculus* (BAB22105), *Homo sapiens* (AAQ88896), *Caenorhabditis elegans* (CAB04533). The tree was construced using the GENEIUOS 4.6.4 software.

4.4.2 Heterologous expression of CpDGAT2 in *S. cerevisiae* H1246

The putative CpDGAT2 identified from the oleaginous fungus *C. purpurea* was expressed in the non-TG forming quadruple mutant *S. cerevisiae* H1246, in which all four genes (*DGA1, LRO1, ARE1 and ARE2*) involved in TG biosynthesis are disrupted (Sandager et al., 2001). Analysis of neutral lipids extracted from wild-type yeast

harboring an empty vector (*S. cerevisiea* INVSC1-pYES2.1/blank) by TLC resulted in detection of five lipid classes as expected (Fig. 4.1). From the top the lipid bands were identified as wax or sterol esters, TGs, FFA, and the two DAG isomers (1.3-DAG and 1.2-DAG). In the mutant yeast with the CpDGAT2 (*S. cerevisiae* H1246-pYES2.1/CpDGAT2) the CpDGAT2 activity was detected as a TG band, which migrated in the same area as the TG from the wild-type. On the other hand, TGs and wax esters were missing on the non-TG forming mutant harboring an empty vector (*S. cerevisiae* H1246-pYES2.1/blank), suggesting that the additional lipid bands present in the *S. cerevisiae* H1246-pYES2.1/CpDGAT2 (wax/sterol esters and TGs) were products of CpDGAT2. Thus, these results clearly indicate that TGs and wax esters were synthesized in *S. cerevisiae* H1246-pYES2.1/CpDGAT2 by CpDGAT2 activity.

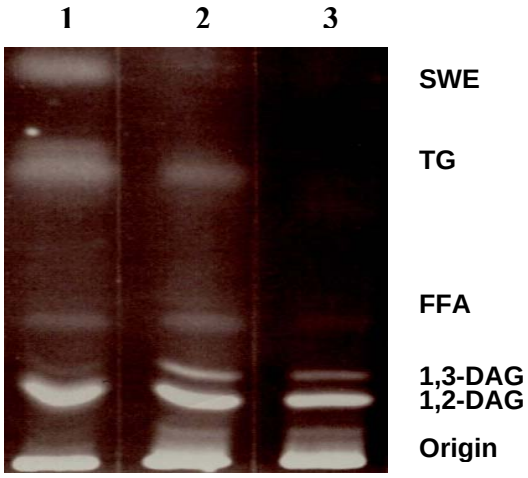


Figure 4.2 Thin-layer chromatography of lipids extracted from yeast transformants. Cells were cultivated in 10ml of DOB minus uracil with 2% galactose for 24 h at 28 ^{0}C. Column 1: *S. cerevisiae* INVSC1 (pYES2.1/blank), column 2: *S. cerevisiae* H1246 (pYES2.1/CpDGAT2), column 3: *S. cerevisiae* H1246 (pYES2.1/blank). Total lipids

were extracted from 1.5g of cells. Abbreviations: DAG-diacylglycerols, FFA-free fatty acids, TG-triacylglycerols, SWE-sterol and wax esters.

4.4.3 Heterologus expression of CpDGAT2 in *S. cerevisiae* H1246 with exogenously administrated ricinoleic acid

In an additional experiment with yeast grown in medium containing 250 μM of ricinoleic acid, neutral lipid analysis of the quadruple mutant yeast expressing CpDGAT2 (*S. cerevisiae* H1246-pYES2.1/CpDGAT2), supported the finding observed in the previous study (chapter 4.4.1). As shown in Figure 4.2, CpDGAT2 restored the ability of *S. cerevisiae* H1246 to synthesize TGs, which migrated in the same area as the wild-type TGs (*S. cerevisiea* INVSC1-pYES2.1/blank). In the *S. cerevisiae* H1246-pYES2.1/blank no TGs were present and therefore, no DGAT activity was detected. In addition to normal TGs, another class of TGs containing ricinoleic acid was detected. TGs containing ricinoleic acid (1OH-TG) were found only in the wild-type yeast (*S. cerevisiea* INVSC1-pYES2.1/blank) and in the mutant expressing the CpDGAT2 (*S. cerevisiae* H1246-pYES2.1/CpDGAT2). The 1OH-TG band migrated in the same area as the 1OH-TG standard and it was also confirmed by gas chromatography (GC) analysis where it was found to contain 33.7% and 30.2% ricinoleic acid in the *S. cerevisiae* H1246-pYES2.1/CpDGAT2 and *S. cerevisiea* INVSC1-pYES2.1/blank sample respectively. These data demonstrated the ability of CpDGAT2 to synthesize hydroxyl-containing TGs.

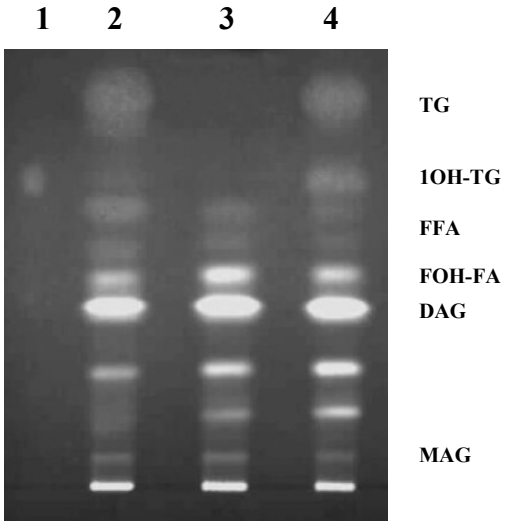


Figure 4.3 Thin-layer chromatography of lipids extracted from yeast transformants. Cells were cultivated in 10ml of DOB minus uracil with 2% galactose for 24 h at 28 ^{0}C. Column 1: 1OH-TG standard, column 2: *S. cerevisiae* INVSC1 (pYES2.1/blank), column 3: *S. cerevisiae* H1246 (pYES2.1/blank), column 4: *S. cerevisiae* H1246 (pYES2.1/CpDGAT2). Total lipids were extracted from 1.5g of cells. Abbreviations: MAG-monoacylglycerols, DAG-diacylglycerols, FOH-FA-free ricinoleic acid, FFA-free fatty acids, 1OH-TG-triacylglycerol with one ricinoleic acid, TG-triacylglycerols.

4.5 Discussion

In this study we demonstrated that the cDNA isolated from the oleaginous fungus *C. purpurea* encodes an acyltransferase with high DGAT activity. Heterologous expression of the putative CpDGAT2 from *C. purpurea* in *S. cerevisiae* H1246 restored TG biosynthesis in this storage lipid-deficient mutant *in vivo* (Fig. 4.1, 4.2). This clearly demonstrated that TG biosynthesis is one of the inherent functions of this putative DGAT2 enzyme isolated from *C. purpurea*. The amount of TGs in the mutant yeast containing CpDGAT2, however, was relatively low in comparison to wild-type

yeast. This could be due to two main factors: First, TG biosynthesis in yeast is catalyzed via two independent pathways that involve the action of four enzymes: an acyl-CoA-dependent acyl-CoA:diacylglycerol acyltransferase encoded by *DGA1* (Oelkers et al., 2002), an acyl-CoA-independent phospholipid:diacylglycerol acyltransferase encoded by *LRO1* (Dahlqvist et al., 2000), and two acyl-CoA:sterol acyltransferases (*ARE1* and *ARE2*) (Sandager et al., 2002) that exhibit a lower level of DGAT activity than the first two enzymes. However, the TG present in lane 2 of Figure 4.1 and 4.2 is the product of a single enzyme (CpDGAT2). Second, *S. cerevisiae* H1246 may not contain very high levels of the substrates preferred by CpDGAT2.

In addition to TGs, CpDGAT2 transformed yeast exhibited very low amounts of wax/sterol esters (Fig. 4.1), supporting the hypothesis that this enzyme possibly possesses bifunctional wax synthase/diaclyglycerol acyltransferase activity, as has been previously described in an Acinetobacter calcoaceticus DGAT2 (Kalscheuer et al., 2004). However, the low amounts of wax esters present suggested the wax synthase activity of CpDGAT2 was limited. Wax synthase activity was not detected when lipids were analyzed from yeast cultures growing in the presence of ricinoleic acid (Fig. 4.2). This however, can partially be explained by the fact that these lipids were developed in a solvent of high diethyl ether, which is suitable for the separation of hydroxyl-containing lipids. The high diethyl ether solvent could result in overlapping of TG fractions with wax/sterol esters.

Analysis of the neutral lipids from CpDGAT2 transformed yeast grown on medium with exogenously administrated ricinoleic acid showed the formation of 1OH-TGs. This indicates that yeast expressing CpDGAT2 can incorporate ricinoleic acid

into TG. The amount of 1OH-TG in CpDGAT2 transformed yeast was higher than the amount of 1OH-TG in wild-type yeast despite the absence of three genes involved in TG biosynthesis. Therefore, although CpDGAT2-containing mutant showed an overall lower activity of TG synthesis than the wild type strain, CpDGAT2 might possess a higher substrate preference towards ricinoleic acid in comparison to the endogenous yeast TG-synthesizing enzymes.

5. SUBSTRATE SPECIFICITY OF CpDGAT2

5.1 Introduction.

Diacylglycerol acyltransferase has a universal role in catalyzing the acyl-CoA-dependent formation of TGs in microorganisms, animals and plants. The major role of TGs is the accumulation of fatty acids as energy source for the cells. In addition, TGs also have dietary and industrial applications. The specific function of TGs, however, depends on the fatty acid composition and sterospecific structure of these molecules. Therefore, the ultimate goal of many studies on TG biosynthesis is to genetically engineer oilseed crops to produce "designed oils" with desirable fatty acid composition and stereospecific structure for industrial or nutritional application.

Diacylglycerol acyltransferase is a key enzyme catalyzing the committed step of TG biosynthesis. To date, several DGAT enzymes have been biochemically characterized for their acyl specificity by *in vivo* and *in vitro* assays. However, no general conclusion has been reached regarding the acyl donor preference of this enzyme. Various *in vitro* studies done on the acyl specificity of DGAT2 from animals

and fungi indicated a preferential utilization towards oleic acid (Cases et al., 2001, Lardizabal et al., 2001) while DGAT2 from tung tree might prefer α-eleostearic acid as the acyl donor (Shockey et al., 2006).

5.2 Objectives and Hypothesis

The first objective of this study was the identification of the preferable acyl donor and acyl acceptor substrates for CpDGAT2 by the microsomal *in vitro* assay. The second objective was to determine whether CpDGAT2 has additional enzymatic activities responsible for the formation of wax monoesters and estolide-TGs.

Our hypothesis is that the DGAT2 enzyme isolated from ricinoleate-producing *C. purpurea* has substrate specificity towards ricinoleic acid. In addition, DGAT2 may possess minor activities of wax and estolide syntheses.

5.3 Materials and Methods

5.3.1 Microsomal preparations extraction and protein quantification

Induced *S. cerevisiae* H1246 cells containing CpDGAT2 were harvested, washed twice with H_2O and homogenized in a buffer containing 400 mM sucrose, 100 mM Hepes-NaOH pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 5 mM EDTA, and 2 mM DTT (Lu et al., 2003). Yeast cells were homogenized using 0.5 mm acid-washed glass beads in a beadbeater (3 X 1 min) at 4 ^{0}C. The homogenate was centrifuged at 10 000 x *g* for 10 min and the supernatant fraction was again centrifuged at 100 000 x *g* for 1 h to obtain the microsomal membrane fraction. This fraction was resuspended in 200 µl of homogenizing medium and the protein concentration was determined by a dye-binding

assay (Bradford, 1976). Eight dilutions containing from 0.1-4 mg/ml of the standard protein (Bovine Serum Albumin) were measured with a spectrophotometer at 595 nm and a standard curve was established. Then a 1:10 dilution of the microsomal fraction was transferred into a clean tube where one ml of Dye Reagent (Bio-Rad) was added. The mixture was then incubated at room temperature for 5 min and transferred to a cuvette with a one cm path length. After the absorbance was measured at 595 nm, the resulted value was applied to a standard protein absorption curve for the determination of protein concentration. Microsomal membrane fractions were then aliquoted and stored at -80 ^{0}C until used.

5.3.2 CpDGAT acyl-donor specificity

For acyl-donor specificity, CpDGAT2 activity was measured as the production of [^{14}C]triacylglycerols from [1-^{14}C]acyl-CoAs and unlabeled 1,2-dioleoyl-*sn*-glycerol. The reaction mixture (0.1 ml) consisted of 0.5-1 µl microsomal preparation, 16.2 µM [1-^{14}C]acyl-CoAs ([1-^{14}C]oleoyl-CoA, [1-^{14}C]linoleoyl-CoA, [1-^{14}C]linolenoyl-CoA, or [1-^{14}C]ricinoleoyl-CoA) (200 000 cpm), and 400 µM 1,2-dioleoyl-*sn*-glycerol dissolved in 20% Tween in a buffer containing 100 mM Tris-HCl pH 7.4, and 20% glycerol as described by He et al. (2006). The assay conditions were also as described by He et al., (2006), except we found that using 20 µg of protein and 5 min incubation time gave higher activity. Assay mixtures were incubated at 25 ^{0}C with shaking, and reactions were stopped by adding 1 ml of isopropanol/CH_2Cl_2 (2:1 v/v).

After the assay was stopped, radiolabeled glycerolipids were isolated by adding 1 ml of stop buffer, 2 ml 0.9% NaCl, and 2 ml of CH_2Cl_2. The mixture was vortexed and

the lower organic phase was transfered to a new glass tube and evaporated to dryness under nitrogen gas. The residue was resuspended in chloroform and subjected to TLC on Silica gel-G plates. TLC plates were developed in hexane:diethyl ether:acetic acid (70:30:1) (v/v/v) for samples containing common acyl-donors and hexane:diethyl ether:acetic acid (70:140:3) (v/v/v) for samples containing [1-^{14}]ricinoleoyl-CoA as the acyl-donor. Developed plates were scanned by a radioimage analyzer (GC-252) to determine the portion of radioactivity incorporated into TG. Radiolabeled bands were then scraped from the plate and the radioactivity of the lipid classes was determined by a Beckman liquid scintillation system (Pillai et al., 1998).

5.3.3 CpDGAT2 acyl-acceptor specificity

To determine the acyl-acceptor specificity of CpDGAT2, microsomal preparations were incubated with 400 μM 1,2-dioleoyl-*sn*-glycerol and 400 μM 1,2-dipalmitoyl-*sn*-glycerol in the reaction mixture described above. A reaction assay lacking DAG substrate was used as a negative control. Each substrate was incubated with 16.2 μM [1-^{14}C]ricinoleoyl-CoA, which was used as the acyl-donor. Lipid extraction and determination of radioactivity was performed according to Pillai et al., (1998).

Two additional assays for the investigation of CpDGAT2 wax synthase and estolide synthetic activities were performed. To investigate estolide synthesis, [1-^{14}C]ricinoleoyl-CoA was incubated with 400 μM triricinolein, while for the wax synthase activity [1-^{14}C]linoleoyl-CoA was incubated with 400 μM 1-hexadecanol. For the visualization of [1-^{14}C]linoleoyl-CoA/1-hexadecanol reaction products, the

extracted lipids were dried, separated by TLC with hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as described by Yen et al. (2005), and scanned by a radioimage analyzer (GC-252). Similarly, the [1-^{14}C]ricinoleoyl-CoA/triricinolein reaction products were separated by TLC with hexane/diethyl ether/acetic acid (70:140:3, v/v/v) and visualized as previously described (Pillai et al., 1998).

5.4 Results

5.4.1 Determination of microsomal protein concentration

Microsomal preparations of CpDGAT2 were isolated according to Lu et al. (2003) and the protein concentration was measured using the Bradford method (Bradford, 1976). Eight dilutions of bovine serum albumin (BSA) (four ranging from 0.1-0.4 mg/ml and four ranging from 1-4 mg/ml) were used for standard curve establishment. Each concentration of BSA was measured in a photospectometer at 595 nm. Absorption values of BSA are shown in the table below (Table 5.1).

The linear relationship (y=ax) between absorbance and concentration is described by the following equation:

$$y = 25.358x \quad (1)$$

Table 5.1 Absorption values of different concentrations of BSA for standard curve establishment.

mg/ml	0.1	0.2	0.3	0.4	1	2	3	4
Absorption (595 nm)	0.001	0.002	0.01	0.016	0.037	0.078	0.112	0.163

where *y* is the concentration in mg/ml and *x* is the absorbance (nm) (Fig. 5.1). For the estimation of the coefficient *a* the following formula was applied:

$$\boldsymbol{a} = (\mathbf{X}'\mathbf{X})^{-1}\,\mathbf{X}'\mathbf{Y},$$

where **X** is the vector of observations for absorbance and **Y** is the vector of observations for concentration (Gujarati 2003). As shown on Figure 5.1 the coefficient of determination (R^2) indicates the variation in our sample is 0.9949. The high R2 value indicates that our standard curve has a strong, linear trend, and therefore we can relatively accurately predict the protein concentration using the absorbance value.

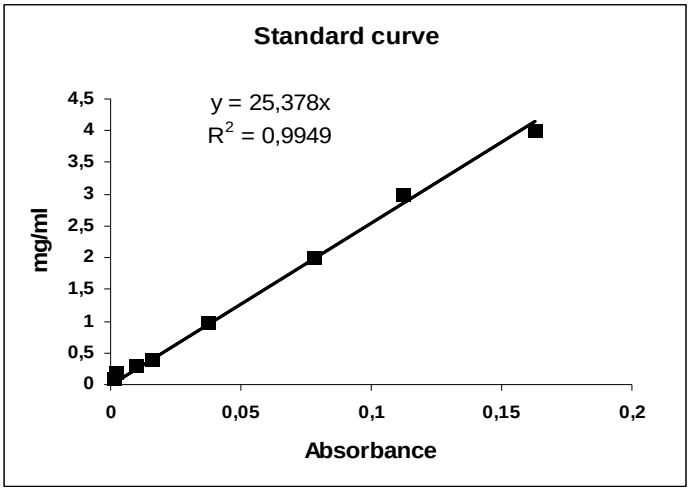


Figure 5.1 Standard curve for protein quantity determination.

5.4.2 CpDGAT acyl donor specificity

The DGAT2 gene identified in the oleaginous fungus *Claviceps purpurea* was expressed in yeast cells to determine the acyl donor specificity. Microsomal preparations from yeast were incubated with 1,2-dioleoyl-*sn*-glycerol and various [1-

^{14}C]acyl-CoAs followed by lipid extraction and analysis of radioactivity. Initially lipid classes were separated by TLC and visualized by a radioimage scanner.

Three radioactive bands identified on the TLC plate corresponded to TG, FFA and the sample application origin (Fig. 5.2). All bands with exception of the origin migrated to different areas on the TLC plate, depending on the solvent used and the fatty acid composition. Thus, in the samples where [1-^{14}C]oleoyl-CoA, [1-^{14}C]linoleoyl-CoA, and [1-^{14}C]linolenoyl-CoA were used as acyl donors, the radioactive bands identified from the top of the plate are TGs, FFA and origin, while when [1-^{14}C]ricinoleoyl-CoA was used as acyl donor the bands identified from the top are TG with one hydroxyl fatty acid (1OH-TG), free ricinoleic acid (FOH-FA) and origin.

In all assay reactions performed, microsomal preparations from yeast transformed with an empty vector (*S. cerevisiae* H1246-pYES2.1/blank) were used as a negative control. As shown in Figure 5.2 CpDGAT2 restored the ability of the mutant yeast to synthesize TGs and utilized all acyl substrates provided. Analysis of radioactivity of TG fractions revealed an increased level of radiolabelled ricinoleic acid-containing TGs, followed by linoleic acid and oleic acid TG fractions. Linolenic acid-TG exhibited the lowest activity (Fig 5.3).

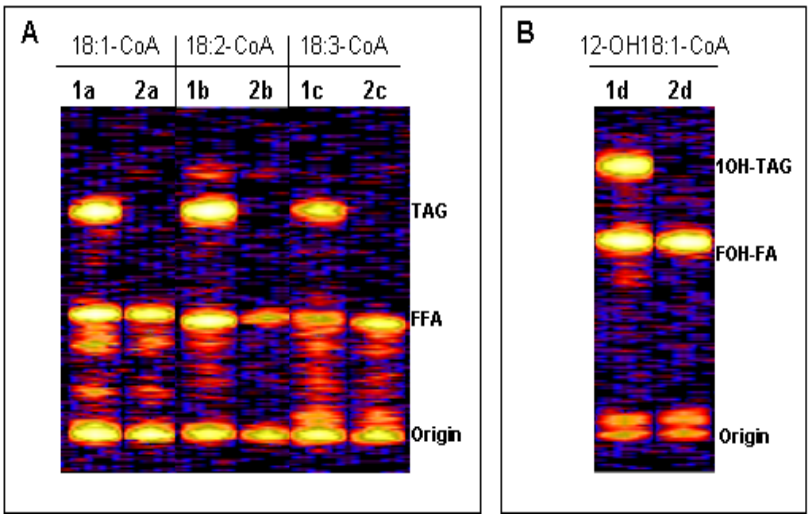


Figure 5.2 TLC analysis of [1-^{14}C]acyl-CoA-containing lipids. A: Lipids separated in hexane:diethyl ether:acetic acid 70:30:1 (v/v/v). B: Ricinoleic acid-containing lipids separated in hexane:diethyl ether:acetic acid 70:140:3 (v/v/v). Lane 1a, 1b, 1c, 1d-*S.cerevisiea* H1246-pYES2.1/CpDGAT2. Lane 2a, 2b, 2c, 2d-*S. cerevisiae* H1246-pYES2.1/blank. TG: triacylglycerol, FFA: free fatty acids, 1OH-TG: triacylglycerol with one ricinoleic acid, FOH-FA: ricinoleic acid.

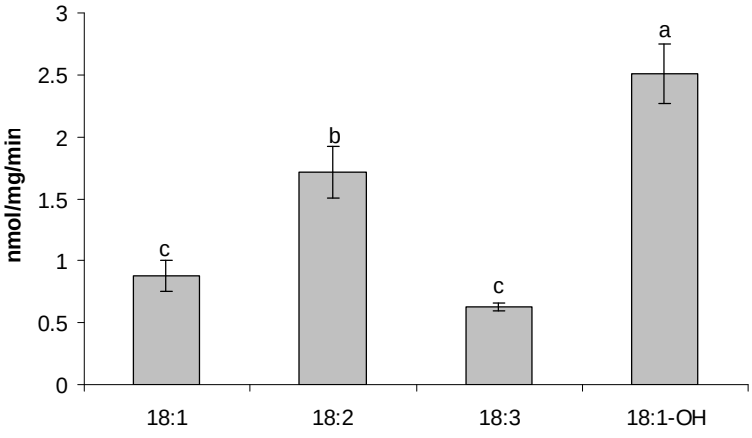


Figure. 5.3 Activity found on TGs after incubation of various [1-^{14}C]acyl-CoAs and 1,2-diolein with *S. cerevisiea* H1246-pYES2.1/CpDGAT2 microsomal preparations. Means

with the same letters are not significant different from each other according to ANOVA Student's t test (P=0.0002, N=3).

Analysis of the radioactivity present in the free fatty acid bands showed that free ricinoleic acid in *S. cerevisiea* H1246-pYES2.1/CpDGAT2 reached the highest value, followed by linoleic, oleic and linolenic acids (Fig. 5.4). The similar activity pattern on free ricinoleic acid was also present in the *S. cerevisiea* H1246-pYES2.1/blank sample. However, radioactivity of oleic, linoleic, and linolenic acids in *S. cerevisiea* H1246-pYES2.1/blank was found to be almost equal.

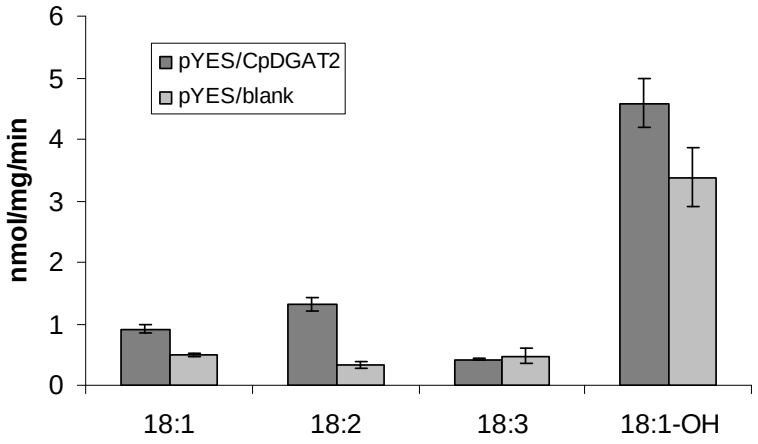


Figure 5.4 Free Fatty Acid radioactivity measured after incubation of various [1-^{14}C] acyl-CoAs and 1,2-diolein with microsomal preparations of pYES2.1/CpDGAT2 and pYES2.1/BLANK transformed yeast.

5.4.3 CpDGAT acyl acceptor specificity

To examine whether the acyltransferase activity of CpDGAT2 depends on the presence of a specific type of DAG, two commercially available DAG molecules (1,2-dioleoyl-*sn*-glycerol and 1,2-dipalmitoyl-*sn*-glycerol) were incubated with the CpDGAT2 preferred acyl-donor substrate ([1-^{14}C]ricinoleoyl-CoA). Three classes of lipids, namely 1OH-TG, FOH-FA and origin (phospholipids/CoAs) were identified based on their migration (Fig. 5.5). All acyl substrates were utilized by CpDGAT2-transformed cells on acyl acceptors compared to yeast with the vector control (*S. cerevisiea* H1246-pYES2.1/blank).

Two additional acyl acceptors, 1-hexadecanol and triricinolein were used in order to investigate the additional activities of CpDGAT2. 1-hexadecanol was used to determine whether CpDGAT2 has wax synthase activity while triricinolein to determine whether CpDGAT2 has estolide activity. However, as shown in Fig. 5.5, no such activity was detected. In order to identify the wax synthase activity, lipids were run in a solvent where wax esters migrated in the top of TLC plate. However, no additional band was present in that area. Bands detected were TG, FFA and at the origin. In case of estolide activity, the TLC plate was developed in a solvent where hydroxyl-containing TGs can be easily visualized. In this case, the bands detected were 1OH-TGs, FOH-FA and origin.

Quantitative determination of radioactivity present in the TG fractions revealed high accumulation of TGs when 1,2-dioleoyl-*sn*-glycerol was used as an acyl acceptor. On the other hand, the amount of radioactivity present in TGs derived from 1,2-dipalmitoyl-*sn*-glycerol was low, similar to that of the negative control (Fig. 5.6).

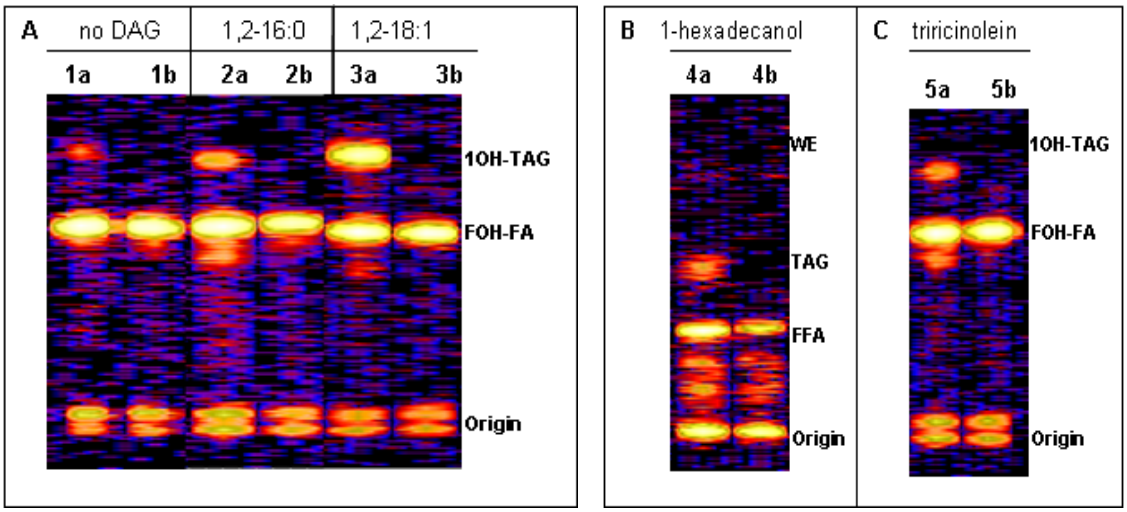


Figure 5.5 TLC separation of TGs containing [^{14}C]acyl-CoA. **A**-Lipids separated in hexane/diethyl ether/acetic acid (70:140:3, v/v/v). 1,2-dioleoyl-*sn*-glycerol and 1,2-dipalmitoyl-*sn*-glycerol were incubated with [^{14}C]ricinoleoyl-CoA and 20 μg of microsomal protein. Lanes 1a, 2a, 3a-*S. cerevisiae* H1246-pYES2.1/CpDGAT2, lane 1b, 2b, and 3b- *S.cerevisiae* H1246-pYES2.1/blank 1OH-TG – TGs containing one ricinoleic acid, FOHFA – free ricinoleic acid. **B**-Lipids separated in hexane/diethyl ether/acetic acid (70:20:1, v/v/v). 1-hexadecanol was incubated with [^{14}C]linoeoyl-CoA and 20 μg of microsomal protein. Lane 4a-*S. cereviciae* H1246-pYES2.1/CpDGAT2, lane 4b-*S. cerevisiae* H1246/pYES2.1. WE – wax esters, TG – triacylglycerols, FFA – free fatty acids. **C**-Lipids separated with hexane/diethyl ether/acetic acid (70:140:3, v/v/v). Triricinoleoin was incubated with [^{14}C]ricinoleoyl-CoA and 20 μg of microsomal protein. Lane 5a-*S. cerevisiae* H1246-pYES2.1/CpDGAT2, lane 5b-*S. cerevisiae* H1246/pYES2.1.

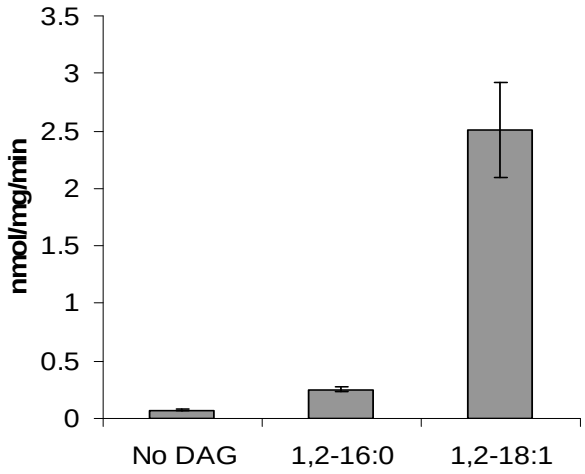


Figure 5.6 Radioactivity (nmol/mg/min) incorporated into TG after incubation of microsomal preparations from yeast transformed with pYES2.1/CpDGAT2 with [1-^{14}C]ricinoleoyl-CoA plus either 1,2-dioleoyl-*sn*-glycerol or 1,2-dipalmitoyl-*sn*-glycerol.

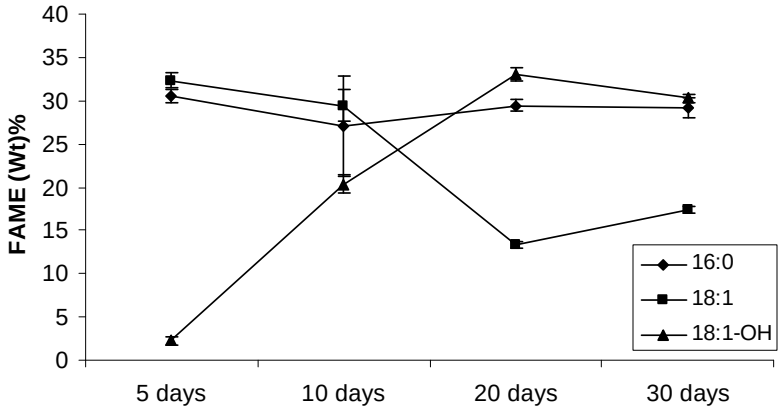


Figure 5.7 Fatty acid profile of 16:0, 18:1, and 18:1-OH as found in TG fractions of sclerotia-like cells of *C. purpurea* after 5, 10, 20, and 30 days of cultivation in ANM medium.

5.5 Discussion

In this study we investigated the acyl substrate specificity of CpDGAT2 by an *in vitro* assay. Microsomal preparations from transformed yeast expressing the CpDGAT2 were incubated with 1,2-dioleoyl-*sn*-glycerol and 1,2-dipalmitoyl-*sn*-glycerol, and various [1-^{14}C]acyl-CoAs. Substrate concentrations used were 16.2 μM of acyl-donor (Weselake et al., 2000, Nykiforuk et al., 2002, Saha et al., 2006) and 400 μM of acyl-acceptor (He et al., 2004, Cases et al., 1998, He et al. 2006, Shokey et al., 2006). The CpDGAT2 assay was initiated by the addition of CpDGAT2 protein, which was applied in the form of microsomal fractions since DGAT2 is strongly associated with the endoplasmic reticulum (Stone, et al. 2009, Shokey et al., 2006, Cao and Hung, 1986). After the assay was stopped, lipids were extracted and separated by TLC. Lipids classes were identified according to their migration points on TLC plates.

Analysis of radioisotope levels incorporated into TGs revealed an enhanced capacity of CpDGAT2 for the utilization of ricinoleic acid as a substrate, followed by linoleic acid and oleic acid, while α-linolenic acid was the least preferred substrate among the acyl-CoAs tested. The increased preference for ricinoleic acid was not an unexpected result, if we take into consideration that ricinoleic acid reaches up to 50% of the total fatty acids in sclerotia from *C. purpurea* (Batrakov and Tolkacher, 1997). In addition to ricinoleic acid, another major fatty acid found in sclerotia is linolenic acid, which reaches up to 35% of the total fatty acids found on TG fractions of *C. purpurea*, while oleic acid is present as a smaller proportion of 20-30% (Batrakov and Tolkacher, 1997). Analysis of radioisotope levels found in free fatty acids showed a large amount of ricinoleoyl was present in the form of free fatty acid. This might be due in part to the

action of a ricinoleoyl-specific phospholipase present in the microsomal preparation, which liberates ricinoleoyl-CoA to its free fatty acid form (Bafor et al., 1991).

Of the two types of DGAT enzymes, DGAT2 has been suggested to preferentially utilize unusual fatty acids for TG formation. For example, compared with tung DGAT1, tung DGAT2 has a higher expression level in developing seeds where α-eleostearic acid is mainly synthesized and tung DGAT2 transformed yeast cells fed with α-eleostearic acid resulted in higher levels of trieleostearin content than tung DGAT1 transformed cells (Shockey et al., 2006). In castor bean seeds, which can accumulate up to 90% of ricinoleic acid, the enzymatic activity of DGAT2 was proven by an *in vitro* assay using diricinolein as the acceptor and ricinoleoyl-CoA as the donor (Kroon et al., 2006). The castor DGAT2 expressed in yeast cells had a strong preference for ricinoleate-containing diacylglycerol substrates (Burgal et al, 2008) while castor DGAT1 shows a greater preference to oleate, *in vitro* (He et al., 2004). DGAT2 exhibits higher expression than the DGAT1 in developing seeds as well (Kroon et al., 2006). Transgenic Arabidopsis revealed that the castor DGAT2 could significantly increase ricinoleic acid production in seeds (Burgal et al., 2008). However, neither castor bean DGAT2 nor any other DGAT2 proteins have been shown, *in vitro*, to preferentially utilize ricinoleic acid or other unusual fatty acids as an acyl donor. To our knowledge, this is the first report describing a preferential utilization of an unusual fatty acid by a DGAT2 protein.

Regarding the acyl acceptor specificity, analysis of the radioactivity incorporated into hydroxyl-containing TGs showed that CpDGAT2 preferentially utilizes 1,2-dioleoyl-*sn*-glycerol as an acyl-acceptor, while activity with 1,2-dipalmitoyl-*sn*-

glycerol was similar to the negative control. The increase in utilization of 1,2-dioleoyl-*sn*-glycerol was an unexpected result if we take into consideration that 18:1 corresponds to 20-30% of the total fatty acids found in TGs while the amount of 16:0 ranges from 25 to 50% of total fatty acids in sclerotia TGs of *C. purpurea* (Batrakov and Tolkacher, 1997). However, we found that the amount of 16:0 and 18:1 in TGs of slerotia-like cells of *C. purpurea* grown in ANM medium changes during the time of cultivation (Fig. 5.7. for details see chapter 7). The low amount of 18:1 at 20 days and 30 days may be due to utilization of this fatty acid by CpFAH for the production of 18:1-OH (Meesapyodsuk and Qiu, 2008). Therefore, the amount of 18:1 and 16:0 incorporated into TGs may reflect the availability of these substrates rather than the CpDGAT2 acyl-acceptor specificity. Similarly, Lardizabal et al., (2001) observed that the *M. rammaniana* DGAT2 acyl-acceptor specificity did not correspond to the fatty acid profile of TGs. They suggested that this may be due to the difference in the solubility of the substrates provided.

To investigate whether CpDGAT2 possesses wax monoester synthetic abilities, 1-hexadecanol was used as an acyl-acceptor. However, although some other DGAT2 enzymes are known to be involved in wax monoester synthesis as well, no wax monoester band was detected in an *in vitro* CpDGAT2 assay. This was similar when mouse DGAT2 was tested *in vitro* for wax synthesis and not such activity was detected (Yen et al., 2005). In general, DGAT2 isoforms appear to function primarily as diacylglycerol acyltransferases, while DGAT1 isoforms may possess multiple acyltransferase activities including wax monoester and wax diester synthesis (Cheng and Russell, 2004).

To assay estolide synthesis activity, microsomal preparations from yeast cells transformed with pYES2.1/CpDGAT2 were incubated with [1-^{14}C]ricinoleoyl-CoA and TG with ricinoleic acid esterified in the *sn*-1, *sn*-2, and *sn*-3 positions. The resulting products were separated on TLC plates developed in hexane/diethyl ether/acetic acid (70:140:3, v/v/v). The point of migration of estolide-TGs on a TLC plate using hexane/diethyl ether/acetic acid (70:140:3, v/v/v) as the mobile phase for neutral lipid separation was expected to lie between the normal TG and FFA bands. However, no additional band between TG and FFA was detected. Additional experiments where triricinolein was incubated with [1-^{14}C]linoleoyl-CoA confirmed the above results. Although the negative control did not showed any radioactive TGs, a weak band corresponding 1OH-TG was detected in CpDGAT2-transformed cells, suggesting the presence of endogenous DAG substrates. These data indicated that CpDGAT2 does not possess estolide-synthesizing capabilities under the assay condition.

6. COEXPRESSION OF CpDGAT2 AND CpFAH IN *SACCHAROMYCES CEREVISIEA* .

6.1 Introduction.

Ricinoleic acid is an 18 carbon hydroxyl fatty acid with a single double bond at carbon 9 and a hydroxyl group attached at position 12. It has been believed for many years that the biosynthesis of this fatty acid involves a hydration process with linoleic acid as the substrate. However, van de Loo et al., (1995) isolated a cDNA from castor bean coding for a fatty acid hydroxylase involved in synthesis of ricinoleic acid,

indicating that biosynthesis of ricinoleic acid in plants is a hydroxylation process, which results in the addition of a hydroxyl group at the 12^{th} carbon of oleic acid. This reaction is catalyzed by a membrane bound enzyme at the *sn-2* position of phosphatidylcholine, using cytocrome b_5 and NADH as cofactors (Bafor et al., 1991). Soon afterwards, another fatty acid hydroxylase gene from the plant *Lesquerella* was identified which is homologous to the castor hydroxylase (Broun et al., 1998).

Our lab recently identified and characterized a fatty acid hydroxylase gene from the ergot fungus *C. purpurea* (CpFAH) involved in biosynthesis of ricinoleic acid (Meesapyodsuk and Qiu, 2008), which, like castor hydroxylase genes, codes for a desaturase-like hydroxylase. To further examine the substrate preference of CpDGAT2 in vivo, this study expressed CpDGAT along with CpFAH in yeast and compared this co-expression with the expression of the native yeast DGAT2 (ScDga1) along with CpFAH in the production of hydroxyl fatty acids.

6.2 Objectives and Hypothesis

The objective of this study was to evaluate by *in vivo* analysis the enzymatic behavior of CpDGAT2 regarding the accumulation of ricinoleic acid when it is coexpressed with the CpFAH and to compare the ricinoleic acid utilization of CpDGAT2 with that of the yeast native DGAT2.

Previous *in vitro* assays have showed that CpDGAT2 preferentially utilizes ricinoleic acid as a substrate. Thus, we hypothesize that CpDGAT2 could preferentially utilize ricinoleic acid *in vivo* as well, thus the yeast expressing CpDGAT2 and CpFAH

could accumulate higher amounts of ricinoleic acid than yeast expressing ScDga1 and CpFAH.

6.3 Materials and Methods

6.3.1 Generation of constructs

The activities of CpDGAT2 and CpFAH were characterized by coexpression of the corresponding full-length cDNAs in *S. cerevisiae* H1246 behind the *GAL1* and *GAL10* promoters, respectively, using the vector pESC-URA (Stratagene). The coding sequence of CpDGAT2 and CpFAH were removed from pYES2.1 as BamHI/NheI and EcoRI fragments respectively by digestion in a reaction containing 5 µl of DNA, 2 µl of 10X digestion buffer, 1 µl of BamHI and 1 µl of NheI restriction enzyme or 2 µl EcoRI, and 11 µl of H_2O according to Ausubel et al., (1995). The fragments were then ligated into the pESC-URA vector digested with the appropriate enzymes using a ligation reaction mixture containing 3 µl insert, 0.5 µl digested vector, 0.5 µl T4 DNA Ligase, 2 µl 5X T4 DNA ligase buffer, and 4 µl H_2O (Ausubel et al., 1995). Initially, the CpFAH open reading frame was PCR amplified with EcoRI sites at the 5' and 3' ends using the 5' GCGAATTCGAAATGGCTTCCGCTACTCC 3' (forward) and 5' GCGAATTCCTACTGAGTCTTCATTGAAATGG 3' (reverse) primers. The 1434 bp fragment was cloned into a pYES2.1/V5-His-TOPO vector and isolated as previously described. The pESC-URA/CpDGAT2/CpFAH construct was compared with the endogenous yeast DGAT2 (ScDga1), which was also coexpressed with CpFAH. The open reading frame of ScDga1 was PCR amplified with the BamHI and NheI restriction sites added to the 5' and 3' positions, respectively. The primers 5'

GGGGATCCAAAATGTCAGGAACATTCAATG 3' (forward) and 5' GGGCTGCTTACCCAACTATCTTCAATTC 3' (reverse) were used to amplified a 1258 base pair fragment, which was cloned into the pCR4-TOPO vector (Invitrogen Canada Inc., Burlington, Ontario). PCR amplification of ScDga1 was conducted in a total reaction volume of 50 µl containing 1.0 µl of 10 µM of each primer, 0.75 µl of 50 mM $MgCl_2$, 5.0 µl of 10X *Pfu* Ultra buffer, 1.0 µl of 10 mM dNTPs, 1.0 µl of *Pfu* Ultra Taq polymerase, and 1.0 µl of yeast cells disrupted with 0.02N NaOH, with the remaining volume consisting of sterile distilled water. The PCR cycling conditions were 10 min at 95 ^{0}C followed by 30 cycles of 30 sec at 95 ^{0}C, 1 min at 53 ^{0}C, and 1 min at 72 ^{0}C and a final extension of 10 min at 72 ^{0}C. The amplified fragment was analyzed by gel electrophoresis and purified as previously described. The pCR4/ScDga1 construct was then sent to PBI, National Research Council of Canada, Saskatoon, SK for sequencing. After sequence analysis the BamHI/NheI ScDga1 fragment was cloned into pESC-URA under *GAL1* promoter followed by cloning of CpFAH under the *GAL10* promoter. The resulting plasmids were introduced into *S. cerevisiae* H1246 cells by lithium acetate-mediated transformation (Gietz and Woods 2002). Two additional plasmids, one containing the CpFAH only and the other consisting of the empty pESC-URA vector, were used as controls.

6.3.2 Fatty acid analysis

Transformed cells were selected for their ability to grow on medium lacking uracil and individual colonies were grown and induced as described above. Galactose induced cells were washed with H_2O and fatty acid methyl esters (FAME) were

prepared as described by Reed et al., (2000). Briefly, the yeast pellet was saponified with 2 ml of 10% (w/v) methanolic KOH in a sealed culture tube at 80 ^{0}C for 2 h. The tubes were then cooled and the non-saponifiable lipids were pre-extracted twice with 2 ml of hexane. The organic phase was neutralized with 2 ml of 50% HCl and the free fatty acids were extracted twice with 2 ml of hexane. For gas chromatography (GC) analysis, fatty acids were transmethylated with 3 N methanolic/HCl at 80 ^{0}C for 2 h and then extracted twice with 2 ml hexane. FAMEs were then dried under nitrogen gas and resuspended in 200 µl of hexane. For identification of hydroxyl containing fatty acids, 100 µl of resuspended FAMEs were treated with 20 µl of N,O-Bis(trimethylsilyl)acetamide/pyridine (1:1) (v/v) (TMS) and the TMS-derivatives were analyzed in a Hewlett-Packard 5890A GC equipped with a DB-23 column (30-m X 0.25-mm) with 0.25-µm film thickness. The column temperature was maintained at 160 ^{0}C for 1 min, then raised to 240 ^{0}C at a rate of 4 ^{0}C/min (Meesapyodsuk et al., 2007).

6.4 Results

6.4.1 Generation of constructs

The yeast expression vector pESC-URA (StraTGene) was used for coexpression studies. This vector contains two multiple cloning sites (MCS), one *GAL1* promoter-MCS-*CYC1* terminator and one *GAL10* promoter-multicloning site-*ADH* terminator, allowing the expression of two genes simultaneously. Four constructs made with this vector (Table 6.1) were transformed into *S. cerevisiae* H1246.

Table 6.1 Constructs used to transform yeast (*S. cerevisiae* H1246).

Construct name	Promoter	
	GAL1	***GAL10***
pESC-URA/blank	-	-
pESC-URA/CpFAH	-	CpFAH
pESCURA/CpDGAT2/CpFAH	CpDGAT2	CpFAH
pESC-URA/ScDga1/CpFAH	ScDga1	CpFAH

To confirm the presence of constructs in transformed cells, the transformants were examined by PCR amplification of transgenes. For the pESC-URA/CpFAH, pESC-URA/CpDGAT2/CpFAH, and pESC-URA/ScDga1/CpFAH constructs, the GAL10 forward and the CpFAH reverse primer were used to amplify the EcoRI cloned CpFAH gene. For the pESC-URA/CpDGAT2/CpFAH construct, the CpDGAT2 forward and reverse primers were used to amplify the BamHI/NheI cloned CpDGAT2 gene and for pESC-URA/ScDga1/CpFAH the ScDga1 forward and reverse primer were used to amplify the BamHI/NheI cloned ScDga1 gene (Fig 6.1). For PCR amplification, single yeast colonies were disrupted in 0.02 N NaOH and the resulting lysate was used as a template. As shown in Fig. 6.1, all colonies with the exception of colony #2 from the pESC-URA/CpFAH construct were found to be positive with the appropriate inserts.

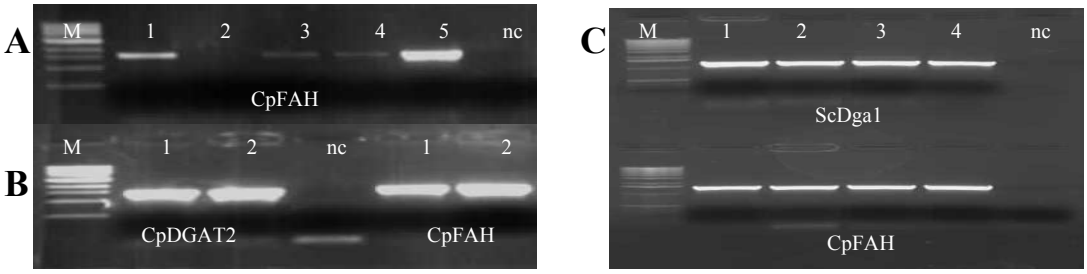


Figure 6.1 Ethidium bromide-stained agarose gel of PCR amplified CpFAH (A),

CpDGAT2/CpFAH (B), and ScDga1/CpFAH (C) genes for confirmation of transformants. Numbers indicate the number of *S. cerevisiea* H1246 colonies checked. M: 1 kb marker, nc: negative control.

6.4.2 Fatty acid analysis

The fatty acid profiles of four constructs were examined. Compared to the negative control (pESC-URA/blank), constructs containing CpFAH (pESC-URA/CpFAH, pEAC-URA/CpDGAT2/CpFAH, pESC-URA/ScDga1/CpFAH) produced three new fatty acids 16:2-9c,12c, 18:2-9c,12c and 12OH-18:1-9c (Fig 6.2). In addition, trace amounts of 12OH-16:1-9c was also observed in CpFAH-transformed yeast cells (Fig. 6.3) by GC analysis of TMS-derivatives. The identification of the hydroxy fatty acids was achieved by comparing the GC retention times of TMS-treated samples with the retention times of standards and non-TMS treated samples.

Comparison of fatty acid profiles from total lipids of pESC-URA/CpFAH, pESC-URA/CpDGAT2/CpFAH and pESC-URA/ScDga1/CpFAH transformed yeast cells revealed that coexpression of CpDGAT2 and CpFAH produced a higher level of ricinoleic acid (at 10.15±0.52% of the total fatty acids) than co-expression of ScDga1 and CpFAH (at 7.38±0.71% of ricinoleic acid). Yeast expressing the CpFAH alone produced 8.13±0.49% of ricinoleic acid (Figure 6.4).

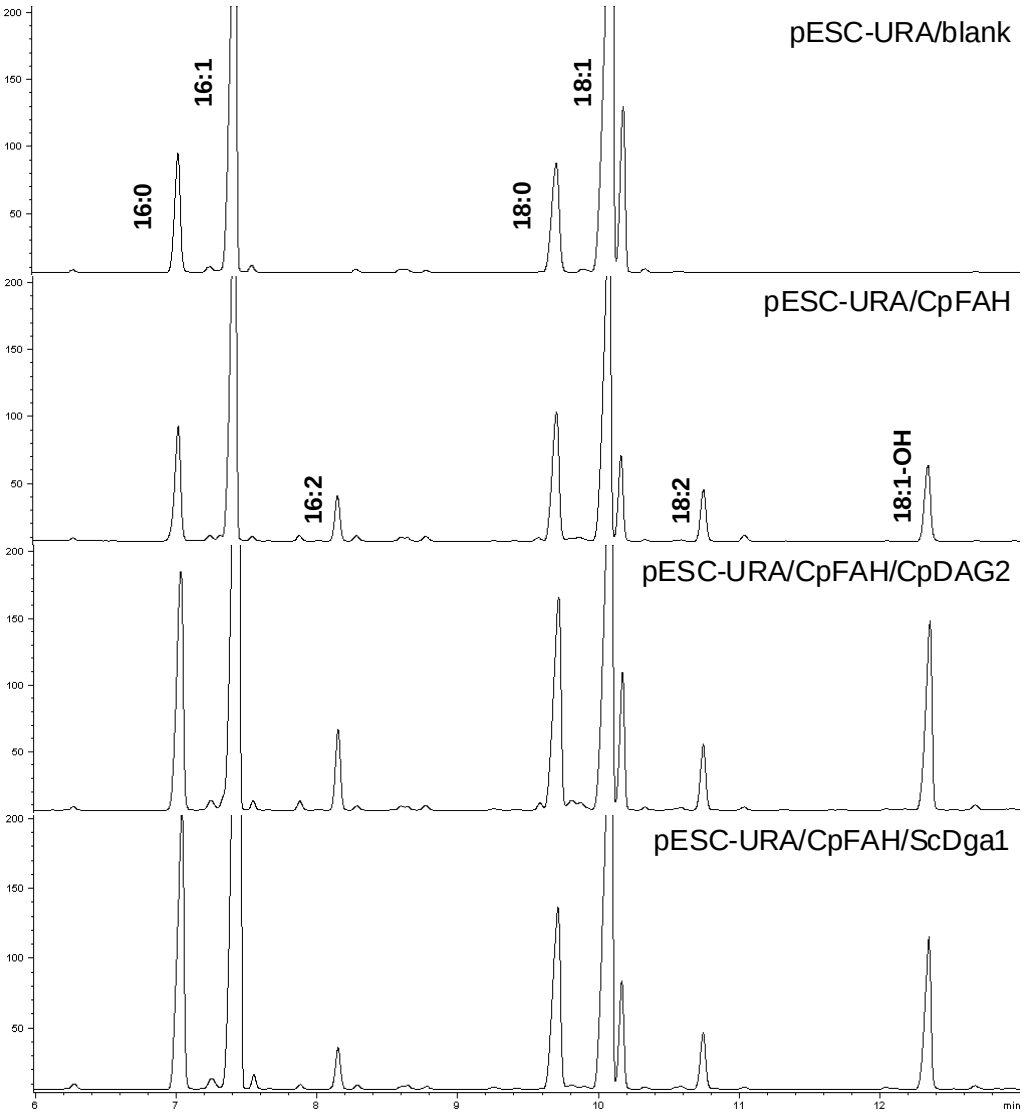


Figure 6.2 Coexpression of CpDGAT2 and CpFAH. GC analysis of FAMEs from *S. cerevisiae* H1246 with the pESC-URA/blank, pESC-URA/CpDGAT2/CpFAH, pESC-URA/CpFAH, and pESC-URA/ScDga1/CpFAH constructs. In the pESC-URA/blank, arrows indicate the major fatty acids present. In the pESC-URA/CpDGAT2/CpFAH, arrows indicate the three additional fatty acid products of CpFAH.

In the negative control with empty vector, the most abundant fatty acid was 18:1-9, reaching up to 40% of the total fatty acids in cells, while no hydroxyl fatty acids

were produced. In the constructs with CpFAH or CpFAH along with DGATs, ricinoleic acid was substantially produced at the cost of oleic acid which was reduced to about 26% on the average. This negative relationship reflects the fact that oleic acid is the substrate of CpFAH for synthesizing ricinoleic acid (Fig. 6.5).

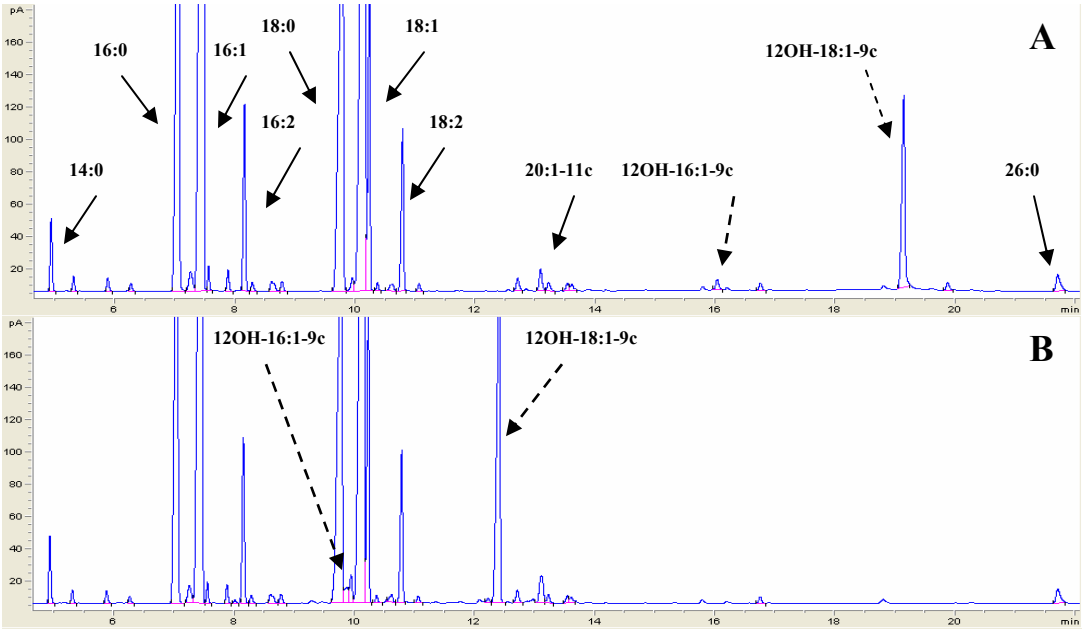


Figure 6.3 GC analysis of FAMEs from *S. cerevisiae*H1246 with the pESC-URA/CpDGAT2/CpFAH construct. A: Non-TMS treated FAMEs. Straight-line arrows indicate the major fatty acids present. Dash-line arrows indicate the position of the hydroxyl fatty acids. B: TMS treated FAMEs.

CpFAH is bifunctional enzyme possessing both desaturase and hydroxylase activitites on oleic acid (Meesapyodsuk & Qiu, 2008). CpFAH coexpressed with CpDGAT2 exhibited a higher percenTGe of hydroxylation than CpFAH coexpressed with ScDga1 or CpFAH alone. The hydroxylation of oleic acid was approximately 3

times higher than the desaturation of oleic acid in pESC-URA/CpDGAT2/CpFAH and pESC-URA/ScDga1/CpFAH, whereas the ratio of desaturation and hydroxylation in CpFAH alone were close to 1.5 (Fig. 6.6). A higher ratio of the hydroxylation versus the desaturation of oleic acid observed in the co-expression of CpFAH and CpDGAT2 (Fig. 6.6) confirmed that CpDGAT2, compared to other related DGAT2s, preferably used ricinoleic acid as a substrate for triacylglycerol synthesis.

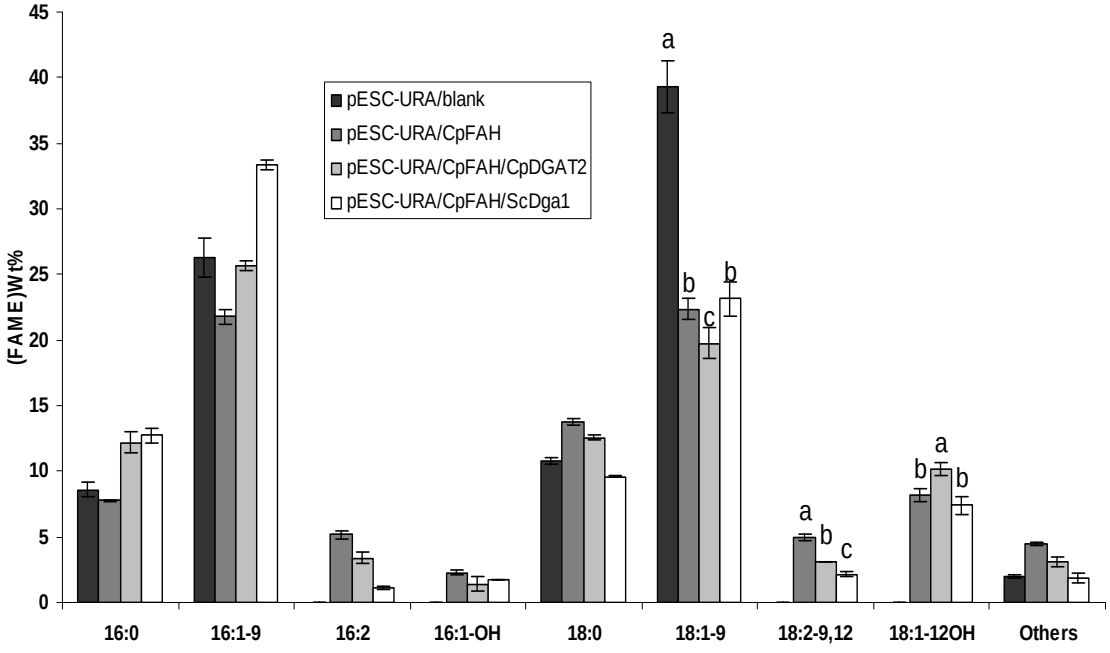


Figure 6.4 Major fatty acids present in pESC-URA/blank, pESC-URA/CpFAH, pESC-URA/CpDGAT2/CpFAH, and pESC-URA/ScDga1/CpFAH transformed *S. cerevisiae* H1246. Experiment was performed in triplicates. Means with the same letters are not significant different according to ANOVA Student's t test (*N*=3, 18:1-9 *P*=0.001, 18:2-9,12 *P*=0.0001, 18:1-12OH *P*=0.002).

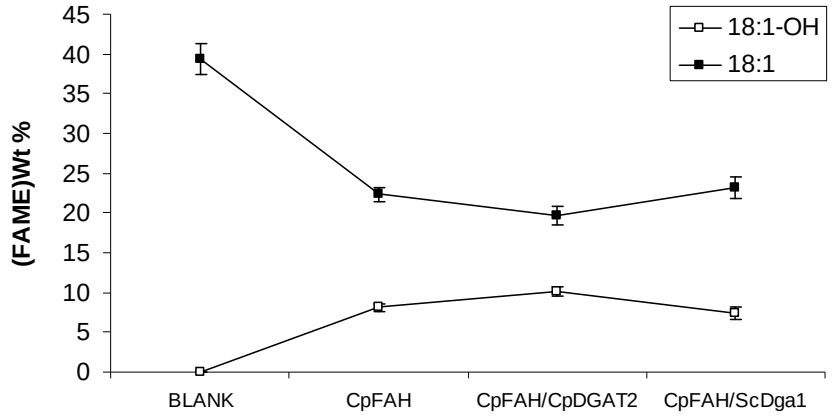


Figure 6.5 Ricinoleic and oleic acids levels in pESC-URA/blank, pESC-URA/CpFAH, pESC-URA/CpDGAT2/CpFAH, and pESC-URA/ScDga1/CpFAH transformed *S. cerevisiae* H1246.

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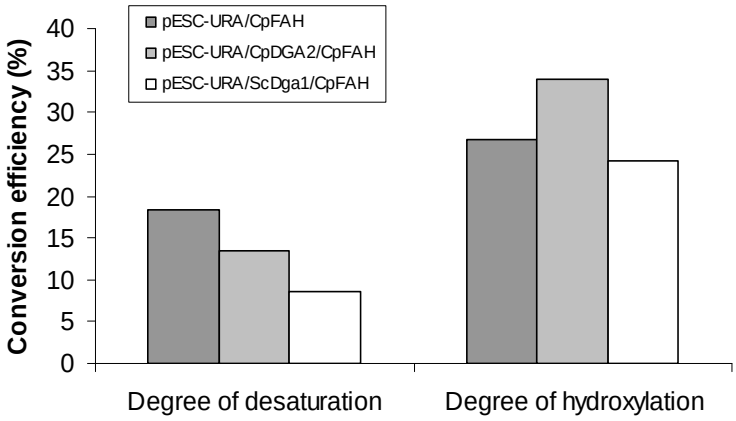

Figure 6.6 Desaturation and hydroxylation activity of CpFAH

6.5 Discussion

In this study we compared *Claviceps* CpDGAT2 with yeast ScDGAT2 (ScDga1) for the enhanced production of ricinoleic acid in yeast. To do this, the two genes were co-expressed with CpFAH in the yeast expression vector pESC-URA that allows the

expression of two genes simultaneously. Four constructs have been used; two for coexpressing CpFAH with CpDGAT2 and ScDga1, respectively, one expressing the CpFAH alone, and the one is an empty vector control. The CpFAH was inserted behind the *GAL10* promoter, while CpDGAT2 and ScDga1 under the control of *GAL1* promoter. All constructs were transformed into *S. cerevisiea* H1246.

Fatty acid analysis revealed that expression of CpFAH alone or along with DGATs all produced three major new fatty acids 16:2-9c,12c, 18:2-9c,12c and 12OH-18:1-9c when compared with the empty vector control (pESC/blank). This confirms previous results that CpFAH is a bifunctional enzyme with hydroxylase/desaturase activities (Meesapyodsuk & Qiu, 2008). The Δ12 desaturase activity introduces a double bond at the Δ12 position of 16:1-8 and 18:1-9, giving 16:2-9,12 and 18:2-9,12, respectively, and the activity of Δ12 hydroxylase predominately introduces a hydroxyl group at the 12 position of oleic acid resulting ricinoleic acid. As well, it can also introduce a hydroxyl group at 12 position of palmitoleic acid giving 12OH-16:1-9 to a lesser extent. Similar results were also observed by Smith and Moon, (2003) with the castor bean hydroxylase when it was expressed in *Arabidopsis*.

Comparison of fatty acid profiles of the four constructs showed an increase in ricinoleic acid content when CpFAH was coexpressed with *C. purpurea* DGAT2 in comparison to that with *S. cerevisiea* DGAT2. This *in vivo* data confirms the above *in vitro* result that CpDGAT2 preferentially uses ricinoleic acid as an acyl donor and supports the result obtained in the ricinoleic acid feeding experiment (study 4), where TLC analysis of neutral lipids showed the presence of 1OH-TG in higher amounts in pYES2.1/CpDGAT2 transformed yeast than in cells carrying pYES2.1/ScDga1.

Variations in the levels of the precursor of ricinoleic acid, 18:1-9c (van de Loo et al., 1995, Meesapyodsuk and Qiu, 2008) were observed among the four constructs. The amount of 18:1-9c in pESC-URA/CpDGAT2/CpFAH yeast was relatively low in comparison to pESC-URA/CpFAH or pESC-URA/ScDga1/CpFAH yeast, indicating a higher conversion efficiency of 18:1-9c to 12OH-18:1-9c by CpFAH in the presence of CpDGAT2. This higher utilization of 18:1-9c by CpFAH is supportive of the preferential incorporation of ricinoleic acid into TG by CpDGAT2. On the other hand, coexpression of DGAT2 genes with CpFAH also affects the bifunctional properties of CpFAH. The construct containing CpDGAT2 exhibits higher hydroxylation activity compared to desaturation activity. The higher degree of hydroxylation versus desaturation in the coexpression of CpDGAT2 and CpFAH reconfirms the notion that CpDGAT2 possesses substrate specificity towards to ricinoleic acid for triacylglycerol synthesis.

7. NORTHERN BLOT ANALYSIS OF *CpDGAT2* AND *CpFAH* IN *C. PURPUREA*.

7.1 Introduction.

C. purpurea displays unique metabolic characteristics when grown under conditions favoring lipid production. Such cultures are accompanied by the formation of sclerotia. The growth of *C. purpurea* sclerotia in anexic cultures can be controlled by the amino acid source as described by Mantel and Nisbet, 1976. Lipid content in anexic cultures reach up to 4% (w/w) in conidiospores, 1% (w/w) in mycelium and 29% (w/w) in sclerotia-like cells (Mantel and Nisbet, 1976). In natural sclerotia, TGs comprise

about 60% of the total lipids and contain a large amount of ricinoleic acid, which in some instance amounts to as much as 50% of the total fatty acids (Batrakov and Tolkachev 1997).

Ricinoleic acid-containing TGs are the major components of ergot oil. The increased lipid content in sclerotia is believed due to the role of sclerotia in the *C. purpurea* life cycle. Sclerotia represent the sTGe in which the fungus survives over the winter. In order to germinate, sclerotia require a period of ~1-2 months at low temperature. Unique morphological and metabolic characteristics of sclerotia confer the fungus to endure this long period of time, and the high lipid content of sclerotia is believed to provide the energy needed for this dormant period.

Lipid composition of sclerotia is unique, in which "normal" TGs with three fatty acids esterified in the *sn-1*, *sn-2* and *sn-3* position are only 1.6% of the total oil. Of seven TG fractions, six are estolide-TGs, a type of TGs esterified with more than three fatty acids. The requirement for the synthesis of this "special" class of TGs is the presence of hydroxyl fatty acids (ricinoleic acid in case of *C. purpurea*) where the hydroxyl group is esterified with an additional fatty acid.

CpFAH and *CpDGAT* are two key genes involved in the biosynthesis and bioassembly of ricinoleic acids in *C. purpurea*. In this study the expression patterns of these two genes along with lipid profiles in different types of *C. purpurea* cells were analyzed.

7.2 Objectives and Hypothesis.

The objective of this study is to analyze the expression patterns of CpDGAT2 and CpFAH in different types of *C. purpurea* cells. In addition, the lipid profile of these cells is also determined to see whether there is any correlation between the gene expression and lipid profile.

CpDGAT2 and CpFAH play a very important role in the synthesis and assembly of ricinoleic acid in *C. purpurea*. Thus, these two genes may be concurrently expressed in the sclerotia-like cells where high levels of ricinoleic acid-containing TGs are accumulated.

7.3 Materials and Methods.

7.3.1 Culture media

Cell samples from three sTGes of the *C. purpurea* life cycle were used for the analysis of CpDGAT2 and CpFAH expression and lipid profile. Sclerotia, mycelia, and conidiospores of *C. purprurea* were obtained in two different media. For sclerotium and conidium production, the 25 day mycelium tissue grown in potato dextrose agar (PDA) medium was used for the inoculation of an amino-nitrogen medium (ANM) consisting of sucrose, 100 g/l; L-asparagine, 5 g/l or other L-amino acids in amounts supplying equivalent nitrogen, e.g. 10 g aspartic acid/l; KH_2PO_4, 0.25 g/l; $MgSO_4 \cdot 7H_2O$, 0.25 g/l; KCl, 0.125 g/l; $FeSO_4 \cdot 7H_2O$, 0.033 g/l; $ZnSO_4 \cdot 7H_2O$, 0.027 g/l as described by Mantel and Nisbet (1976). The pH was adjusted to 5.2 with concentrated NaOH and the medium containing 2% (w/v) agar was sterilized at 120 ^{0}C for 20 min. Sclerotium-like cells were collected from the ANM medium after 5, 10, 20, 25, and 30

days' cultivation. Conidiospores were collected after 40 days' cultivation. Mycelium cells were collected after 25 days' cultivation in PDA medium. Samples were stored at -80 ^{0}C until used.

7.3.2 Lipid analysis

Lipids extracted from sclerotium, mycelium, and conidium cells were analyzed for TG and ricinoleic acid content. The quantitative analysis of TGs from sclerotia-like cells (5, 10, 20, 25, and 30 days old), mycelium cells (25 days old), and conidial cells was performed by thin-layer chromatography (TLC) and gas chromatography (GC). Briefly, lipids extracted from 5 mg of freeze-dried cells were subjected to TLC separation according to Aitzetmuller et al., (1992). Next, the identified TG spot was scraped from the plate and used for the preparation of methyl-esters for GC analysis as previously described by Meesapyodsuk et al., (2007) with 200 μg of 17:0 added as an internal standard.

Additionally, the TG molecules obtained from sclerotia-like cells of *C. purpurea* were analyzed using ^{1}H-NMR. The ^{1}H-NMR spectra was obtained using a Brunker spectrometer (PBI, National Research Council of Canada, Saskatoon, SK) with a 5-mm TXI probe (500 MHz) using deuterated chloroform as a solvent.

7.3.3 Total RNA extraction

Total RNA was extracted from the collected sclerotium, conidium and mycelium samples for the analysis of CpDGAT2 and CpFAH expression levels. Total RNA was isolated with TRizol (Invitrogen Canada Inc., Burlington, Ontario) according to the

manufacturer's instructions. Briefly, fungal samples were ground with liquid N_2 using a mortar and pestle and then transferred into a 1.5 ml eppendorf tube. For sclerotium and mycelium samples, 1 ml of TRizol per 1 mg of sample was added into the tube, while for spores 1 ml of TRizol per 5 x 10^6 cells was used. Following homogenization, insoluble material was removed by centrifugation at 12,000 x g for 10 min at 4 ^{0}C. The resulting supernatant was then transferred into a clean tube and incubated for 5 min at 24 ^{0}C to permit the complete dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform per 1 ml of TRizol was added and the tube was shaken vigorously by hand for 15 sec, followed by incubation for 3 min at 24 ^{0}C. The mixture was then centrifuged at 12,000 x g for 15 min at 4 ^{0}C and the resulting upper aqueous phase was transferred into a clean tube. Total RNA was precipitated from the aqueous phase by mixing with 0.5 ml of isopropyl alcohol per 1 ml of TRizol used for the initial homogenization. Samples were then incubated at 24 ^{0}C and centrifuged at 12,000 x g for 10 min at 4 ^{0}C. The supernatant was removed and the RNA pellet was washed twice with 1 ml of 75% ethanol. The washed RNA was briefly air-dried for 5 min and dissolved in 100 μl of DEPC-treated water. The quantity of extracted RNA was determined by spectrophotometry. Total RNA was stored at -80 ^{0}C until used.

7.3.4 Denaturing gel electrophoresis and alkaline transfer

For Northern hybridization, 7 μg of total RNA was separated by gel electrophoresis and transferred onto a nylon membrane according to Ausubel et al., (1995). A standard denaturing agarose gel consisting of 1.2% agarose, 43.5 ml DEPC-treated H_2O, 1.5 ml 37% formaldehyde and 10 ml 10 x MOPS was used for the

separation of RNA samples, which were prepared in a mixture containing 20 µl of 1.25X RNA loading buffer (62.5 µl of 100% formamide, 12.5 µl of 10 x MOPS, 24 µl of 37% formaldehyde, and 1 µl of 4% bromophenol blue), 0.4 mg/ml ethidium bromide, and 7 µg of total RNA in a final volume of 20 µl. Prior to electrophoresis, the RNA mixture was denatured at 65 ^{0}C for 10 min followed by cooling on ice immediately. Then, samples were loaded into 50 µl wells and ran at 65 V for 45 min in 1 x MOPS buffer. After electrophoresis, the gel was rinsed with DEPC-treated H_2O and the RNA was visualized under UV light (SYNGENE Bio Imaging System). RNA was then transferred from the denaturing gel onto a nylon membrane. The transfer system was built by placing the followings in sequence from bottom to top: a glass tray partially filled with 20 x SSC, a glass support placed on the tray like a bridge, a long strip of Whatman 3MM paper on the support with its edges immersed in the 20 x SSC buffer, followed by two pieces of Whatman 3MM paper in the shape of the gel. The DEPC-H_2O washed gel was placed on the Whatman 3MM paper with the well openings facing down, a nylon membrane in the shape of the gel was placed on the gel, followed by three pieces of Whatman 3MM paper and finally a thick pile of paper towels. The system was stabilized by placing a glass plate and a 0.5 kg weight on the top of the pile of paper towels. All Whatman 3MM papers were soaked in the 20 x SSC buffer prior to being placed in the system. The nylon membrane initially was soaked into DEPC-treated H_2O and after placement on the gel, its upper side was flooded with 20 x SSC buffer. The transfer system was run overnight. Following the transfer, the nylon membrane was removed from the transfer system and washed with 2X SSC. Then, the membrane was dried for 1 h at room temperature and the transferred total

RNA was cross-linked to the membrane using a CL-1000 Ultraviolet Crosslinker apparatus. The membrane was then stored at 4 ^{0}C until used.

7.3.5 Generation of CpDGAT2 and CpFAH probes

Probes used for Northern hybridization consisted of a 1314 bp and a 428 bp cDNA fragment corresponding to portions of the CpDGAT2 and CpFAH genes, respectively. For the generation of the CpDGAT2 probe, primers 5' CAGGATCCGAGATGGCAGCCGTCCAAGTC 3' (forward) and 5' ACGCTGCTCAAGACAAGATCTGCAGCTC 3' (reverse) were used to amplify the fragment by PCR, using the pESC-URA/CpDGAT2 plasmid as a template. The PCR reaction for CpDGAT2 probe amplification was performed as described above (Section 6.3.1). For the generation of the CpFAH probe, primers 5' CGCTCTTTGGAAGACTGCTC 3' (forward) and 5' ATCCCCAATTCCAGTTCTA 3' (reverse) were used for PCR amplification of the fragment. Since the coding region of CpFAH shares approximately 80% identity at the nucleotide level with the Δ12 desaturases CpDesX and CpDes12 (Meesapyodsuk and Qiu, 2008), the primers used to amplify the CpFAH probe targeted the 3' untranslated region (UTR) of this gene where the similarity with CpDesX and CpDes12 is reduced to approximately 50%. Prior to CpFAH probe amplification, total RNA from sclerotia-like cells of *C. purpurea* was used for the synthesis of cDNA. The newly synthesized cDNA was used as the template for the synthesis of the CpFAH 3' UTR in a mixture consisted of 2.5 μl of 10 x PCR buffer, 0.75 μl of 50 mM $MgCl_2$, 0.5 μl of 10 mM dNTPs, 0.5 μl of 10 μM of each primer, 0.25 μl of Taq polymerase, 3.0 μl of cDNA, and 17.5 μl of H_2O. The PCR

conditions consisted of an initial denaturation at 95 ^{0}C for 5 min followed by 30 cycles of 95 ^{0}C for 2 sec, 56 ^{0}C for 1 min, and 72 ^{0}C for 50 sec, and a final extension at 72 ^{0}C for 5 min. After analysis by gel electrophoresis, the amplified fragment was extracted from the gel and cloned into pCR2.1-TOPO (Invitrogen Canada Inc., Burlington, Ontario), and the resulting plasmid was transformed into competent *E. coli* cells according to the manufacturer's instructions. Plasmids were extracted from *E. coli* cells and sent to PBI, National Research Council of Canada, Saskatoon, SK for sequencing. The sequenced pCR2.1-TOPO plasmid containing the CpFAH 3' UTR was then used as the template for PCR amplification of the CpFAH probe. The PCR reaction was performed using the temperature profile described above. The PCR mixture consisted of 5 µl of *Pfu* PCR buffer, 1 µl of *Pfu* Ultra Taq polymerase, 1 µl of 10 mM dNTPs, 1 µl of 10 µM CpFAH forward primer, 1 µl of 10 µM CpFAH reverse primer, 1 µl of 1/10 diluted plasmid, and 40 µl of H_2O. Both fragments (CpDGAT2 and CpFAH 3'UTR) were quantified by gel electrophoresis using 5 µl of a 1 kb marker as a standard.

Probes were radioactively labeled using the Random Primers DNA Labeling System (Invitrogen Canada Inc., Burlington, Ontario). Twenty-five nanograms of amplified fragments diluted in 20 µl of distilled H_2O were denatured by heating for 5 min in a boiling water bath followed by cooling on ice. The labeling reaction mixture, consisting of 2 µl of dATP, 2 µl of dGTP, 2 µl of dTTP, 15 µl of random primers buffer mixture, 5 µl of [α-^{32}P]dCTP (10 µCi/µl), 3 µl of H_2O, and 1 µl of Klenow fragment, was then added to the tube containing the denatured DNA fragment . The mixture was then incubated at 25 ^{0}C for 2 h and the radiolabeled probes were purified

by passing through a Sephadex G-50 DNA Grade column according to the manufacturer's instructions. Initially the column was washed with 400 µl of TE buffer. The probe was then loaded followed by the addition of 400 µl of TE buffer. Finally, the purified probe was collected by elution with 400 µl of TE buffer. The radioactivity level of the probe was checked using a Geiger counter, after which the probe was denatured at 100 ^{0}C for 5 min. The probe was then cooled on ice and immediately used for the hybridization procedure.

7.3.6 Hybridization, washing and detection

Initially the membrane was prehybridized in a volume of 50 µl/cm^2 of prehybridization solution consisting of 5 x SSPE, 50% formamide, 5 x Denhardt's solution, 0.5% SDS and 100 µg/ml of denatured salmon sperm DNA at 42 ^{0}C for 4 h as previously described (Davis et al., 1986). Prior to use, the prehybridization solution was preheated at 42 ^{0}C. Following prehybridization the membrane was hybridized in a hybridization oven with slow rotation at 42 ^{0}C for 24 h. The hybridization buffer used was the same as the prehybridization buffer except that 1/3 of the purified labeled probe was added. After hybridization, the membrane was washed twice for 10 min with 2 x SSPE followed by a 20 min wash with 0.2 x SSPE, 0.1% SDS at 50 ^{0}C for the removal of nonspecifically bound probe (Davis et al., 1986). The membrane was then checked for radioactivity using a Geiger counter, placed into an autoradiography plate and stored at – 80 ^{0}C until film development.

For radioactivity detection a Kodak Biomax MR Film (20.3 cm x 25.4 cm) was used. The film was manually developed after four days of exposure using manual

developer and manual fixer solutions (White Mountain Imaging, Salisbury, NH) according to the manufacturer's instructions. Briefly, in a red light room the film was washed for 5 min with the manual developer solution followed by washing with water for 1 min. Then the film was washed with manual fixing solution followed by a final rinse with water for 30 min. All washing steps were carried out in separate trays at room temperature.

7.4 Results

7.4.1 Lipid analysis of different types of cells of *C. purpurea*

Prior to expression analysis of CpDGAT2 and CpFAH in different types of cells, their products, ricinoleic acid and ricinoleic acid-containing TGs were determined in these samples. For TG analysis, neutral lipids isolated from 5, 10, 20, 25, 30 days' sclerotia-like cells, 25 days' mycelium cells, and conidial spores were separated by TLC and the TG fractions were quantified by GC. As shown in Figure 7.1, separation by thin-layer chromatography revealed that TGs were the major class of neutral lipids detectable in all types of cells of *C. purpurea*, as has previously been observed by Batrakov and Tolkacher (1997). Quantitative analysis (Table 7.1) of TG fractions indicated that sclerotia-like cells generally had a higher TG content than other types of cells, and conidial spores had the lowest amount of TGs. The highest amount of TGs was obtained from sclerotia-like cells of 20 days' culture in ANM medium. The total amount of fatty acids from TG fractions in 20 days-old sclerotia was 415.3±55.4 μg, while mycelia and conidial spores amounted to 65.96±1.1 and 9.11±0.5 μg,

respectively,. However, sclerotia-like cells of 5 days' culture in ANM had lower fatty acid content than mycelium cells.

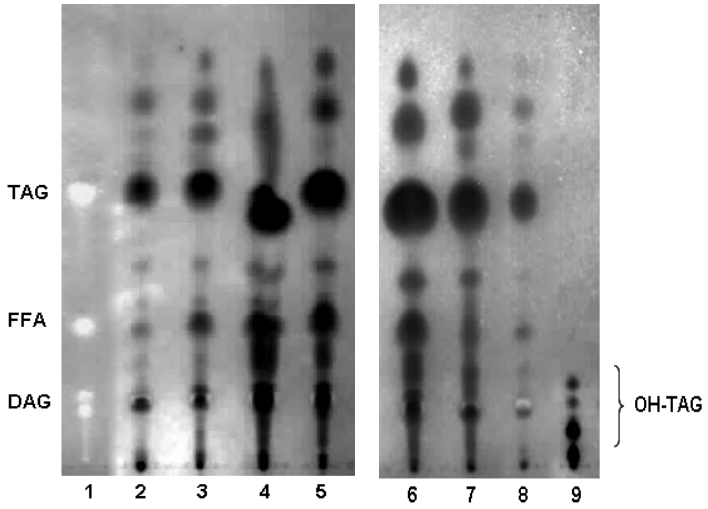

Figure 7.1 TLC analysis of total lipids from sclerotia-like, mycelium and conidial cells of *C. purpurea*. Lanes: 1, 9, standard; 2, 5 day; 3, 10 day; 4, 20 day; 5, 30 day; 6, 25 day cultures of sclerotia-like cells grown in ANM; 7, 25 day cultures of mycelia grown in PDA; 8, conidia (40 day culture grown in ANM). Total lipids were extracted from 5 mg of dried weight cells and the plate was developed in hexane/diethyl ether/acetic acid (70:30:1, v/v/v). Lipids were visualized by iodine. Standards (18:0, OH-TGs) were visualized by primuline solution.

Table 7.1 Fatty acid quantity of TGs isolated from 5 mg dried weight of different types of *C. purpurea* cells.

Days of cultures Type of tissue Media	5-days Sclerotia (ANM)	10-days Sclerotia (AMN)	20-days Sclerotia (ANM)	25-days Sclerotia (ANM)	30-days Sclerotia (ANM)	40-days Conidia (ANM)	25-days Mycelium (PDA)
Total FA quantity (µg)	20.38±3.2f	117.4±18.1d	415.4±55.4a	195.8±26b	155.8±16.22c	9.11±0.5g	65.96±1.1e
18:1-OH quantity (µg)	0.45±0.1d	23.83±3.3c	137.0±15.8a	50.58±6.8b	47.07±4.2b	-	-

Means with the same letters are not significant different according to ANOVA Student's t test (P=0.0001, N=3)

Fatty acid analysis of the TG fractions indicated that ricinoleic acid was one of the major fatty acids in TG fractions of sclerotia-like cells, especially at the 20 and 25 day sclerotium culture, and it reached up to 33 and 32% of the total TG fatty acids, respectively (Fig. 7.2). On the other hand, TG fractions from conidial spores and mycelium cells did not contain any hydroxyl fatty acids and their major fatty acids were oleic acid and palmitic acid, which accounted for more than 30% of the total TG fatty acids.

Analysis of the cell total fatty acids revealed a slightly different pattern (Fig. 7.3). Linoleic acid is the most abundant fatty acid in mycelium cells and conidial spores, followed by oleic acid and palmitic acid. In addition, ricinoleic acid had the highest value in 10 day-old sclerotia-like cells, followed by the values in 20, 25 and 30 days' slcerotium cells, while in TG fatty acid profile, ricinoleic acid reached its maximum value in 20 day cultures, followed by 25, 30 and 10 day cultures.

As shown in Fig. 7.1, TLC analysis indicated that ricinoleic acid-containing TG fractions in sclerotium cells migrated to the spot close to the normal TG standard tristearin on the plate developed by hexane/diethyl ether/acetic acid (70/30/1, v/v/v) as the mobile phase, different from the spots of the hydroxyl fatty acid-containing TG standard castor bean oil. This indicated that these ricinoleic acid-containing TG fractions might not be common triglycerides with hydroxyl fatty acids. To find out the authentic nature of these TG fractions, the TG samples were examined by NMR analysis. Identification of ^{1}H-NMR spectra of the TG fraction and comparison of it with

that of artificially synthesized TG-estolides from lesquerella oil (Isbell and Cermak, 2002) revealed some intriguing features for the hydroxyl TGs in sclerotium cells. (Fig 7.4). In the ^{1}H-NMR spectra of normal TGs containing 18:1-OH, the free OH group of ricinoleic acid is expected to resonate at 3.61 ppm (Batrakov and Tolkacher 1997). However, as shown in Figure 7.4, this free OH signal was not present, suggesting that the hydroxyl group of ricinoleic acid present in the TGs of sclerotium cells is esterified with an additional fatty acid. In addition, ^{1}H-NMR analysis of TG samples from sclerotium cells, identified a methine signal at 4.86-4.81 ppm, which is typical for estolide structure.

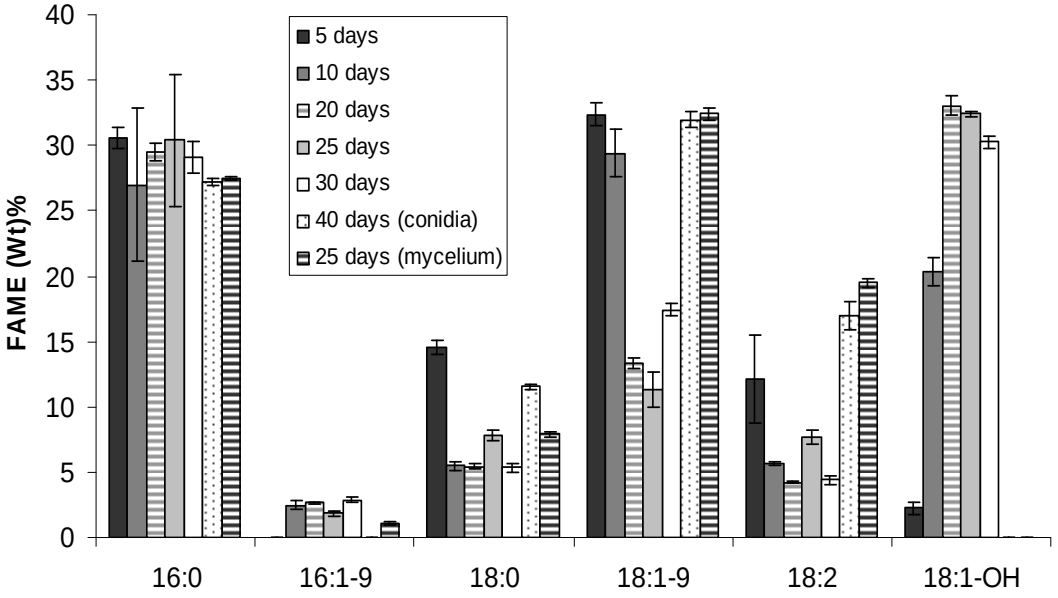

Figure 7.2 Fatty acid profiles of TGs from 5, 10, 20, 25 and 30 day sclerotia-like cells, 25 day mycelium, and conidial cultures of *C. purpurea*

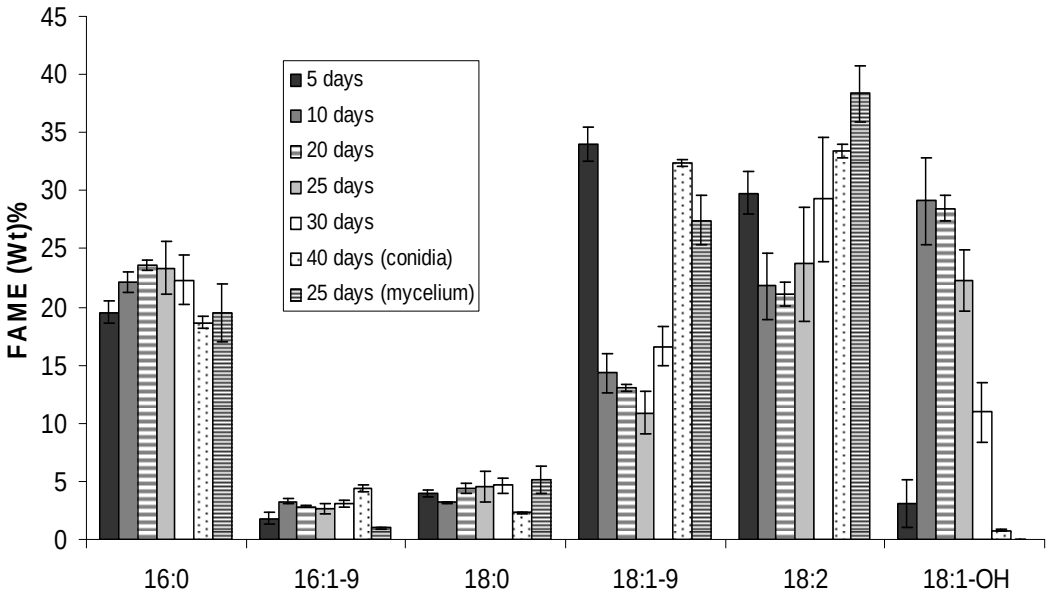

Figure 7.3 Total fatty acid profiles from 5, 10, 20, 25 and 30 day sclerotia-like cells, 25 day mycelium, and conidial cultures of *C. purpurea*

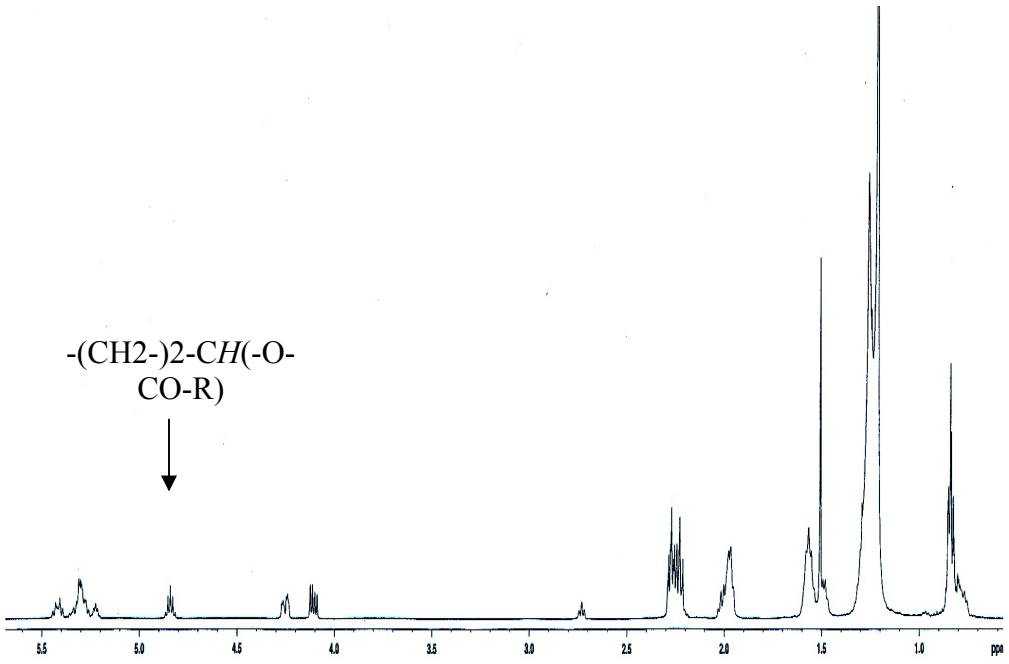

Figure 7.4 ^{1}H-NMR (500 MHz) spectra of TG fractions from *C. purpurea* sclerotia-like cells. Sample was run in deuterated chloroform ($CDCl_3$).

7.4.2 CpDGAT2 and CpFAH mRNA expression analysis

Northern blot analyses were performed to determine the expression levels of CpDGAT2 and CpFAH in different types of *C. purpurea* cells. Two separate membranes, one loaded with the total RNA from 5, 10, 20, and 30 day-old sclerotia-like cells and the other loaded with the total RNA from 25 day sclerotia, 25 day mycelia, and conidial spores, were hybridized with CpDGAT2 and CpFAH probes, respectively. Membranes were initially hybridized with the CpDGAT2 probe, then stripped and rehybridized with the CpFAH probe. As shown in Figure 7.5, CpDGAT2 is expressed in all sclerotia-like cells tested, with the highest expression in 20 day cultures, followed by 30, 10 and 5 day cultures. CpDGAT2 is also highly expressed in mycelium cells. CpFAH, like CpDGAT2, was expressed in all sclerotia-like cells, however, unlike CpDGATs, its expression was highest in 10 day-old cells, followed by 20 day-old cells. Low but detectable levels of the expression were observed in 5 and 30 day sclerotia-like cell cultures. Conversely, CpFAH was not expressed in mycelium or conidial cells (Fig. 7.5).

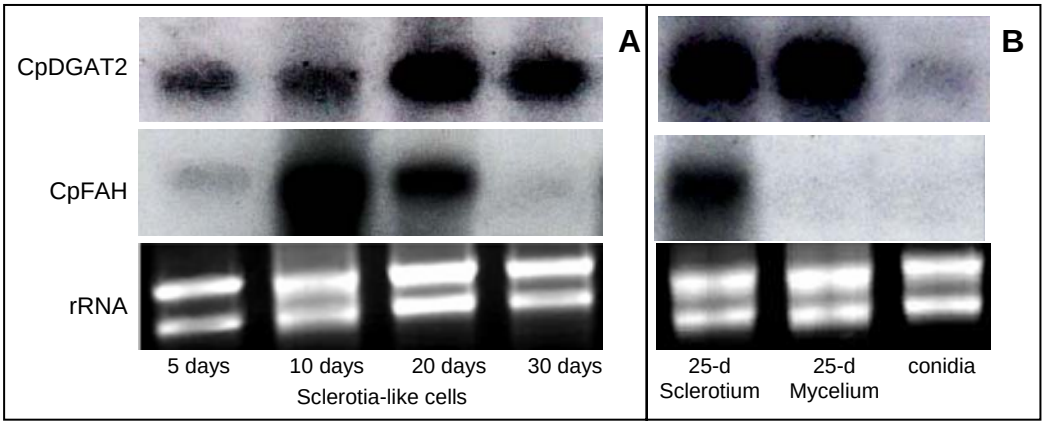

Figure 7.5 Northern blot expression analysis of CpDGAT2 and CpFAH genes. Approximately 7 μg of total RNA was loaded in each lane. A, RNA gel blot analysis of

CpDGAT2 and CpFAH transcripts from 5, 10, 20, and 30 days' sclerotia-like cells. B, RNA gel blot analysis of CpDGAT2 and CpFAH transcripts from 25 days' sclerotia, 25 days' mycelia and conidial spores.

7.4 Discussion

The higher amount of TGs found in sclerotia-like cells of *C. purpurea* was not an unexpected result, as the sclerotium is an energy storage tissue, and is at the sTGe of the life cycle when the fungus survives over the winter (Corbett et al., 1975). The amount of TG fractions in sclerotia-like cells reaches a maximum value in 20 day cultures, followed by 25 and 30 day cultures, with the lowest amount being harvested from 40 day cultures. The low amount of TGs in 40 day cultures may be due to the fact that *C. purpurea* does not follow the normal life cycle in the anexic culture condition. Natural sclerotia germinate to form stroma with ascospores. By contrast, the sclerotia-like cells at the late sTGe differentiate into conidia spores in anexic cultures and the *C. purpurea* sexual sTGe cannot be obtained under the culture condition (Esser and Tudzynski, 1978).

The presence of ricinoleic acid in the TG-like spot of a TLC plate was an unexpected result, since ricinoleic acid-containing TGs such as those in castor oil possess a higher polarity than TGs with common fatty acids and migrate to a different position. ^{1}H-NMR analysis of this ricinoleate TG fraction did not detect the hydroxyl signal that is normally present in the spectra of hydroxyl TGs. The lack of a free hydroxyl group in the ricinoleate TGs implies that the hydroxyl group of ricinoleic acid in TG fraction might be used up, for instance, for acylation with an additional fatty acid

forming estolide, a TG molecule with more than three fatty acids. This could explain the change of the molecule in polarity on the TLC plate. Furthermore, the presence of the estolide methine signal in the ^{1}H-NMR spectra of this TG fraction indicates the TG is indeed esterified with an additional fatty acids at the OH group of ricinoleic acid. The TG estolide has been identified in oil extracted from sclerotia of *C. purpurea* (Batrakov and Tolkacher 1997), however, the migration point of these lipids in silica chromatographic plates is unclear. Our data indicated that estolides migrate to the place close to TG with common fatty acids.

With the exception of 5 day sclerotium cultures, ricinoleic acid was the major fatty acid in the sclerotia-like cells. No ricinoleic acid was detected in mycelium cells and conidial spores. Although mycelium grown in ANM was reported to produce a low amount of ricinoleic acid (Mantel and Nisbet, 1976), mycelium tissues grown in PDA as in our experiments did not produce this fatty acid. It was also found that mycelium cells did not differentiate into sclerotia-like cells regardless of the number of days of cultivation in this medium.

The high amount of ricinoleic acid found in the TG fraction of 10, 20, 25, and 30 day sclerotia-like cells implies that both CpFAH and CpDGAT2 must be expressed in these tissues. Northern analysis indicated that expression pattern of two genes is indeed well correlated with the lipid accumulation pattern. CpFAH is solely expressed in sclerotium cells where ricinoleic acid is accumulated. No CpFAH transcripts were detected in mycelia and conidial spores where no ricinoleic acid is produced. CpDGAT2 is more widely expressed in cell types examined with exception of conidial spores where the expression level is low. Increased expression of CpDGAT2 was

observed in lipid samples with high TG levels. This is especially true with sclerotium cells where a higher level of ricinoleate TGs is accumulated. The high expression of CpFAH in 10 day sclerotium cells, along with increased expression of CpDGAT2 and accumulation of ricinoleic acid TG fractions in 20 and 30 day sclerotium cultures is consistent with notion that CpDGAT2 and CpFAH are two key enzymes involved in the biosynthesis and bioassembly of ricinoleic acid in *C. purpurea*. These data also support the above findings in the *in vitro* assay that CpDGAT2 preferentially utilizes ricinoleic acid as a substrate for TG synthesis.

8. GENERAL SUMMARY AND CONCLUSSIONS

TG is a glyceride in which a glycerol is acylated with three fatty acids, which represents an efficient energy source for living organisms. The last step in TG biosynthesis is catalyzed by DGAT1 or DGAT2, two microsomal enzymes that acylate the last fatty acid in the *sn*-3 position of a DAG molecule. Although both enzymes catalyze the same reaction, they share no or little homology at the primary sequence. Previous research suggested that of the two DGATs, DGAT2 might be the enzyme responsible for the synthesis of TGs containing unusual fatty acids (Shockey et al., 2006, Burgal et al., 2008). The oleaginous fungus *C. purpurea* can produce up to 50% of ricinoleic acid in its sclerotium cells, an unusual hydroxyl fatty acid with various industrial applications including lubricants, functional fluids, ink, paints, coatings, nylons and resins. In this study, a putative DGAT2 isolated from the fungus *Claviceps purpurea* was biochemically characterized by *in vitro* and *in vivo* assays regarding its role in the assembly of this unusual fatty acid into triglyceride.

Functional expression of the CpDGAT2 in *S. cerevisiae* H1246 was able to restore the ability of this TG deficient mutant to synthesize TGs. Analysis of neutral lipids from the CpDGAT2-expressing yeast fed with ricinoleic acid revealed that CpDGAT2 had ability to utilize this hydroxyl fatty acid for TG synthesis. In a coexpression experiment where the ricinoleate synthetic gene *CpFAH* (Meesapyodsuk & Qiu, 2008) was expressed along with CpDGAT2 or yeast DGAT2 (ScDga1), an increased accumulation of ricinoleic acid was observed when CpFAH was coexpressed with CpDGAT2.

For the *in vitro* investigation of substrate preference for the CpDGAT2, microsomal preparations of *S. cerevisiae* H1246 expressing the CpDGAT2 were incubated with various radiolabeled [1-^{14}C]acyl donors and two commercially available acyl acceptors. Analysis of radioactive TGs revealed that among [1-^{14}C]acyl donors tested, ricinoleoyl-CoA was mostly utilized by CpDGAT2, followed by linoleoy-CoA, oleoyl-CoA and α-linolenoyl-CoA. Regarding the acyl acceptor specificity, *sn*-1,2-diolein appeared to be the preferable substrate for CpDGAT2 compared to *sn*-1,2-dipalmitin. Additional assays performed using fatty alcohol and triricinolein as acyl acceptors indicated that CpDGAT2 do not have activities of wax monoester and estolide synthesis.

In the Northern blot study, the expression of CpDGAT2 and CpFAH in different types of cells of *C. purpurea* were investigated. Mycelia, conidiospores and various sTGes of sclerotia-like cells from *C. purpurea* were analyzed for the RNA expression of CpDGAT2 and CpFAH as well as for the lipid and fatty acid contents. It was found that the expression of CpFAH was detected only in sclerotium cells, and the highest expression occurred in 10 day-old cultures. Similar pattern was observed with ricinoleic acid content, i.e. the hydroxyl fatty acid only occurred in sclerotium cells with the highest being at 10-20 day-old cultures. CpDGAT2 expression was detected in all samples tested with the higher expression found in sclerotium and mycelium cells. The expression pattern of CpDGAT2 in sclerotia-like cells was in good agreement with the amount of TGs accumulated and the amount of ricinoleic acid present in TGs. These results are indicative of the correlative relationship between the CpDGAT2 expression and the TG content and the hydroxyl TG production.

Collectively, this research has demonstrated that CpDGAT2 is a key enzyme involved in the assembly of ricinoleic acid into triacylglycerol in *C. purpurea*. It clearly showed substrate preference for this unusual hydroxyl fatty acid, which renders CpDGAT2 as the first *in vitro* characterized DGAT2 enzyme with substrate specificity towards unusual fatty acids. Future studies will be needed to gain further insight into the properties and function of this enzyme. This may include optimization of the wax and estolide assay conditions to confirm the negative results we obtained, site-directed muTGenesis to determine key amino acids for the substrate specificity, and knockout of CpDGAT2 in *C. purpurea* to reveal biological function of this enzyme.

9. REFERENCES

Abo-Hashema, K.A., Cake, M.H., Power, G.W. and Clarke, D. (1999). Evidence for triacylglycerol synthesis in the lumen of microsomes via a lipolysis-esterification pathway involving carnitine acyltransferases. The Journal of Biological Chemistry, 274: 35577-35582.

Achaya, K.T. (1971) Chemical derivatives of castor oil. Journal of American Oil Chemistry Society, 48: 758-763

Aitzetmuller, K., Yaonian, X., Warner, G. and Gronheim, M. (1992) High-performance liquid chromatography investigations of stillingia oil. Journal of Chromatography, 603: 165-173

Alam, M., Gilham, D., Vance, D.E. and Lehner, R. (2006). Mutation of F417 or L420 in the lipid biding domain decreases the activity of triacylglycerol hydrolase. Journal of Lipid Research, 47: 375-383.

Alberts, A.W. and Vagelos, P.R. (1968). Acetyl-CoA carboxylase, I. Requirement for two protein fractions. Proceedings of the National Academy of Science of the United States of America, 59: 561-568.0

Alvarez, H.M. and Steinbuchel, A. (2002). Triacylglycerols in prokaryotic microorganisms. Applied Microbiological Biotechnology, 60: 367-376.

Athenstaedt, K. and Daum, G. (2006). The life cycle of neutral lipids: synthesis, storage and degradation. Cellular and Molecular Life Sciences. 63: 1355-1369.

Athenstaedt, K. and Daum, G. (1997) Biosynthesis of phosphatidic acid in lipid particles and endoplasmic reticulum of *Saccharomyces cerevisiae*. Journal of Bacteriology. 179: 7611–7616.

Athenstaedt, K., Weys, S., Paltauf, F. and Daum, G. (1999a) Redundant systems of phosphatidic acid biosynthesis via acylation of glycerol-3-phosphate or dihydroxyacetone phosphate in the yeast *Saccharomyces cerevisiae*. Journal of Bacteriology, 181: 1458–1463.

Athenstaedt, K., Zweytick, D., Jandrositz, A., Kohlwein, S.D. and Daum, G. (1999b) Identification and characterization of major lipid particle proteins of the yeast *Saccharomyces cerevisiae*. Journal of Bacteriology, 181: 6441–6448.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Stuhl, K. (1995) Current protocols in molecular biology. Vols 1, 2 and 3. New York:Wiley.

Badami, R.C. and Patil, K.B. (1980). Structure and occurrence of unusual fatty acids in minor seed oils. Progress in Lipid Research, 19: 119-153.

Bafor, M., Smith, M.A., Jonsson, L., Stobart, K. and Stymne, S. (1991) Ricinoleic acid biosynthesis and triacylglycerol assembly in microsomal preparations from developing castor-bean (*Ricinus communis*) endosperm. Biochemical Journal, 280: 507-514.

Baldock, C., Rafferty, J.B., Stuitje, A.R., Slabas, A.R. and Rice, D.W. (1998). The X-ray structure of *Escherichia coli* enoyl reductase with bound NAD+ at 2.1 Å resolution. Journal of Molecular Biology, 284: 1529-1546.

Batrakov, S.G. and Tolkacher, O.N. (1997) The structures of triacylglycerols from sclerotia of the rye ergot *Claviceps purpurea* (fries) Tul. Chemistry and Physics of Lipids, 86: 1-12.

Bharucha, K.E. and Gunstone, F.D. (1957). Vegetable oils. Part VI. The component acids of ergot oil. Journal of Chemical Society, 610-614.

Blokker, P., Schouten, S., van de Ende, H., de Leeuw, J.W., Hatcher, P.G. and Damste, J.S.S. (1998). Chemical structure of algaenans from the fresh water algae *Tetraedron minimum*, *Scenedesmus communis* and *Pediastrum boryanum*. Organic Geochemistry, 29: 1453-1468.

Bodalo-Santogo, A., Bastida-Rodriguez, J., Maximo-Martin, M.F., Montiel-Morte, M.C. and Murcia-Almago, D.M. (2005) Enzymatic biosynthesis of ricinoleic acid estolides. Biochemical Engineering Journal, 26: 155-158.

Borch-Jensen, C. and Mollerup, J. (1996). Determination of vernolic acid content in the oil of *Euphorbia lagasceae* by gas and supercritical fluid chromatography. Journal of the American Oil Chemists' Society, 73: 1161-1164.

Bradford, M.M. (1976) A rapid and sensitive method for the detection of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Broun, P., Boddupalli, S. and Somerville, C. (1998). A bifunctional oleate 12-hydroxylase: desaturase from *Lesquerella fendleri*. Plant Journal, 13: 201-210.

Broun, P. and Somerville, C. (1997). Accumulation of ricinoleic, lesquerolic, and densipolic acids in seeds of transgenic *Arabidopsis* plants that express a fatty acid hydroxylase cDNA from castor bean. Plant Physiology, 113: 933-942.

Burgal, J., Shockey, J., Lu, C., Dyer, J., Larson, T., Graham, I. and Browse, J. (2008). Metabolic engineering of hydroxyl fatty acid production in plants: RcDGAT2 drives dramatic increases in ricinoleate levels in seed oil. Plant Biotechnology Journal, 6: 1-13.

Cahoon, E.B., Carlson, J.T., Ripp, K.G., Schweiger, B.J., Cook, G.A., Hall, S.E. and Kinney, A.J. (1999). Biosynthetic origin of conjugated double bonds: Production of fatty acids components of high-value drying oils in transgenic soybean embryos. Proceedings of the National Academy of Science of the United State of America, 96: 12935-12940.

Cahoon, E.B. and Kinney, A.J. (2004). Dimorphecolic acid is synthesized by the coordinate activities o two divergent Δ^{12}-oleic acid desaturase. Journal of Biological Chemistry, 279: 12495-12502.

Cahoon, E.B., Ripp, K.G., Hall, S.E. and Kinney, A.J. (2001). Formation of conjugated delta8,delta10-double bonds by delta12-oleic-acid desaturase-related enzymes: biosynthetic origin of calendic acid. The Journal of Biological Chemistry, 276: 2637-2643.

Cahoon, E.B., Ripp, K.G., Hall, S.E. and McGonigle, B. (2002a). Transgenic production of epoxy fatty acids by expression of a cytochrome P450 enzyme from *Euphorbia lagascae* seed. Plant Physiology, 128: 615-624.

Cahoon, E.B., Schnurr, J.A., Huffman, E.A. and Minto, R.E. (2002b). Fungal responsive fatty acid acetylenases occur widely in evolutionary distant plant families. Plant Journal, 34: 671-683.

Cao, Y.Z. and Huang, A.H.C. (1986) Diacylglycerol acyltransferase in maturing oil seeds of maize and other species. Plant Physiololy, 82: 813–820.

Cases, S., Smith, S.J., Zheng, Y., Myers, H.M., Lear, S.R., Sande, E., Novak, S., Collins, C., Welch, C.B., Lusis, A.J., Erickson, S.K. and Farese Jr, R.V. (1998). Identification of a gene encoding an acyl CoA:diacylglycerol acyltranferase, a key enzyme in triacylglycerol synthesis. Proceedings of the National Academy of Sciences of the United States of America, 95: 13018-13023.

Cases, S., Stone, S.J., Zhou, P., Yen, E., Tow, B., Lardizabal, K.D., Voelker, T. and Farese, R.V. Jr. (2001). Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members. Journal of Biological Chemistry, 276: 38870-38876.

Chen, H.C., Smith, S.J., Tow, B., Elias, P.M. and Farese, R.V. Jr. (2002). Leptin modulates the effect of acyl CoA:diacylglycerol acyltransferase deficiency on murine fur and sebaceous glands. The Journal of Clinical Investigation, 109: 175-181.

Cheng, J.B. and Russell, W.D. (2004). Mammalian wax biosynthesis II Expression cloning of wax synthase cDNAs encoding a member of the acyltransferase enzyme family. Journal of Biological Chemistry, 279: 37798-37807.

Chenna, R., Sugawara, H., Koide, T., Lopez, R., Gibson, T.J., Higgins, D.G. and. Thompson, J.D. (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Research, 31: 3497-3500.

Corbett, K., Dickerson, A.G. and Mantle, P.G. (1975). Metabolism of the germinating sclerotium of *Claviceps purpurea*. Journal of General Microbiology, 90: 55-68.

Cuperus, F.P., Boswinkel, G. and Derksen, J.T.P. (1996). Supercritical carbondioxide extraction of *Dimorphotheca pluvialis* oil seeds. Journal of American Oil Chemists' Society, 73: 1675-1679.

Dahlqvist, A., Stahl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H. and Stymne, S. (2000). Phospholipid:diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proceedings of the National Academy of Science of the United States of America, 97: 6487-6492.

Dauk, M., Lam, P., Kunst, L. and Smith, M.A. (2007). A FAD2 homologue from *Lesquerella lindheimeri* has predominantly fatty acid hydroxylase activity. Plant Science, 172: 43-49.

Davis, L.G., Dibner, M.D. and Battey, J.F. (1986) Basic methods on molecular biology, Elsevier.

Dayer, J.M., Chapital, D.C., Kuan, J.W., Mullen, R.T., Turner, C., McKeon, T.A. and Pepperman, A.B. (2002). Molecular analysis of a bifunctional fatty acid conjugase/desaturase from tung. Implications for the evolution of plant fatty acid diversity. Plant Physiology, 130: 2027-2038.

Dettman, J.R., Jacobson, D.J., Turner, E., Pringle, A., Taylor, J.W. (2003). Reproductive isolation and phylogenetic divergence in Neurospora: comparing methods of species recognition in a model eukaryote. Evolution, 57: 2721-2741.

Duncan, R.A., Sullivan, R. and Alderman, S.C. (2002). *Claviceps purpurea* var.*spartinae* var.nov.an ergot adapted to the aquatic environment. Mycotaxon, 81: 11-25.

Donaldson, R.P. (1977) Accumulation of free ricinoleic acid in germinating castor bean endosperm. Plant Physiology, 59: 1064-1066

Douhan, G.W., Smith, M.E., Huyrn, K.L., Westbrook, A., Beerli, P. and Fisher, A.J. (2008). Multigene analysis suggests ecological speciation in the fungal pathogen *Claviceps purpurea*. Molecular Ecology, 17: 2276-2286.

Esser, K. and Tudzynski, P. (1978). Genetics of the ergot fungus *Claviceps purpurea*. Theoretical and Applied Genetics, 53: 145-149.

Farmer, E.E. (1994). Fatty-acid signaling in plants and their associated microorganisms. Plant Molecular Biology, 26: 1423-1437.

Fisher A.J., DiTomaso, J.M. and Gordon, T.R. (2005). Conidial morphology and ecological characteristics as diagnostic tools for identifying *Claviceps purpurea* from salt-marsh habitats. Canadian Journal of Plant Pathology, 27: 389-395.

Gardes, M. and Bruns, T.D. (1993). ITS primers with enhanced specificity for basidiomycetes--application to the identification of mycorrhizae and rusts. Molecular Ecology, 2: 113-118.

Gaumann, E.A. (1952). The fungi. Hafner Publishing Co, N.Y.

Gietz, R.D. and R.A. Woods. (2002) Transformation of yeast by the Liac/SS carrier DNA/PEG method. Methods in Enzymology, 350: 87-96

Gujarati, D.N. (2003) Basic econometrics 4th Edition.

Gunstone, F.D. (1954). Fatty acids part II. The nature of the oxygenated acids present in *Vernonia anthelmintica*. Journal of the Chemical Society, 1611-1616.

Hamberg, M., Liepinsh, E., Otting, G. and Griffiths, W. (1998). Isolation and structure of a new galctolipid from oat seeds. Lipids, 33: 355-363.

Hammond, E.G., Duvick, D., Wang, T., Dodo, H. and Pittman, R.N. (1997). Survey of the fatty acid composition of peanut (*Arachis hypogeae*) gerplasm and characterization of their epoxy and eicosanoic acids. Journal of the American Oil Chemists' Society, 74: 1235-1239.

Harwood, J. (1996). Recent advances in the biosynthesis of plant fatty acids. Biochimica et Biophysica Acta, 1301: 7-65.

Hatanaka, T., Shimizu, R. and Hildebrand, D. (2004). Expression of a *Stokesia laevis* epoxygenase gene. Phytochemistry, 65: 2189-2196.

He, X.H., Chen, G.O., Lin, J.T. and McKeon, T.A. (2006) Diacylglycerol acyltransferase activity and triacylglycerol synthesis in germinating castor seed cotyledons. Lipids Journal, 41: 281-285.

He, X.H., Turner, C., Chen, G.Q., Lin, J.T. and McKeon, T.A. (2004). Cloning and characterization of a cDNA encoding diacylglycerol acyltransferase from castor bean. Lipids, 39: 311-318.

Heath, R.J., White, S.W. and Rock, C.O. (2001). Lipid biosynthesis as a target for antibacterial agents. Progress in Lipid Research, 40: 467-497.

Hobbs, D.H., Lu, C. and Hills, M.J. (1999). Cloning of a cDNA encoding diacyglycerol acyltransferase from *Arabidopsis thaliana* and its functional expression. FEBS Letters, 452: 145-149.

Hofmann, K. (2000). A superfamily of membrane-bound O-acyltransferases with implications for wnt signaling. Trends in Biochemical Sciences, 25: 111-112.

Isbell, T.A. and Cermak, S.C. (2002) Synthesis of triglycerides estolides from lesquerella and castor oil. Journal of the American Oil Chemists' Society, 79: 1227-1233.

Iwabuchi, M., Kohno-Murase, J. and Imamura, J. (2003). Δ12-oleate desaturase-related enzymes associated with formation of conjugate trans-Δ11, cis-Δ13 double bonds. Journal of Biological Chemistry, 278: 4603-4610.

Jiang, R.W., Hay, M.E., Fairchild, C.R., Prudhomme, J., Roch, K.L., Aalbersberg, W. and Kubanek, J. (2008). Antineoplastic unsaturated fatty acids from Fijian macroalgae. Phytochemistry, 69: 2495-2500.

Johnson, P.E., Rawsthorne, S. and Hills, M.J. (2002). Export of acyl chains from the plastids isolated from embryos of *Brassica napus* (L). Planta, 215: 515-517.

Kalscheuer, R., Luftmann, H. and Steinbuchel, A. (2004). Sunthesis and novel lipids in *Saccharomyces cerevisiae* by heterologous expression of an unspecific bacterial acyltransferase. Applied and Environmental Microbiology, 70: 7119-7125.

Kayden, H.J., Senior, J.R. and Mattson. F.H (1967). The monoglyceride pathway of fat absorption in man. The Journal of Clinical Investigation, 46: 1695–1703.

Kennedy, E.P. (1961) Biosynthesis of complex lipids. Federation Procceding, 20: 934-940.

Kleiman, R., Plattner, R.D. and Spencer, F.G. (1977). *Alchornea cordifolia* seed oil: A rich source of a new C_{20} epoxide, (+)*cis*-14,15-epoxy-*cis*-11-eicosenoic acid. Lipids, 12: 610-612.

Koufopanou, V., Burt, A., Szaro, T. and Taylor, J.W. (2001). Gene genealogies, cryptic species, and molecular evolution in the human pathogen Coccidioides immitis and relatives (Ascomycota, Onygenales). Molecular Biology and Evolution, 18: 1246-1258

Kren, V., Rezanka, T. and Rehacek, Z. (1985). Occurrence of ricinoelic acid in submerged cultures of various *Claviceps* sp. Experientia, 41: 1476-1477.

Kroon, J.T., Wei, W., Simon, W.J. and Slabas, A.R. (2006). Identification and functional expression of a type 2 acyl-CoA:diacylglycerol acyltransferase (DGAT2) in

developing castor bean seeds which has high homology to the major triglyceride biosynthetic enzyme of fungi and animals. Phytochemistry, 67: 2541-2549.

Kuldau, G A. Liu, J.-S., White, J F., Siegel, M R. and Schardl, C L. (1997) Molecular systematics of Clavicipitaceae supporting monophyly of genus *Epichloe* and form genus Ephelis. Mycologia, 89: 434-441

Langdon, R.F.N. (1954) The origin and differentiation of *Claviceps* species. University of Queensland Papers Bot. 3: 61-68

Lardizabal, D.K., Mai, T.J., Wagner, W., Wyrick, A., Voelker, T. and Hawkins, J.D. (2001) DGAT2 in a new diacylglycerol acytransferase gene family. The Journal of Biological Chemistry, 276: 38862-38869.

Lee, H.S., Pho, J.R., Sim, C.J. and Shin, J. (2003). New acetylenic acids from the sponge of the genus Stellata. Journal of Natural Products, 66: 566-568.

Lee, M., Lenman, M., Banas, A., Bafor, M., Singh, S., Schweizer, M., Nilsson R., Liljenberg, C., Dalhqvist, A., Sjodahl, S., Green, A. and Stymne, S. (1998). Identification of non-heme diiron proteins that catalyze triple bond and epoxy group formation. Science, 280: 915-918.

Lee, A.K.Y., Lau, A.P.S., Cheng, J.Y.W., Fang, M. and Chan, C.K. (2007). Source identification analysis for the airborne bacteria and fungi using a biomarker approach. Atmospheric Environment, 41: 2831-2843.

Lehner, R. and Kuksis, A. (1993). Triacylglycerol synthesis by an sw-l,2(2,3)-diacylglycerol transacylase from rat liver and intestinal microsomes. Journal of Biological Chemistry, 268: 8781 - 8786.

Li, X.C., Jacob, M.R., ElSohly, H.N., Nagle, D.G., Smillie, T.J., Walker, L.A. and Clark, A.M. (2003). Acetylenic acids inhibiting azole-resistant *Candida ablicans* from *PenTGonia gigantifolia*. Journal of Natural Products, 66: 1132-1135.

Lin, J.T., Woodruff, C.L., Lagouche, O.J., McKeon, T.A., Stafford, A.E., Goodrich-Tanrikulu, M., Singleton, J.A. and Haney, C.A. (1998). Biosynthesis of tricylglycerols containing ricinoleate in castor microsomes using 1-acyl-2-oleoyl-sn-glycerol-3 phosphocholine as the substrate of oleoyl-12-hyroxylase. Lipids, 33: 59-69.

Liu, Y. and Longmore, R.B. (1997). Dietary sandalwood seed oil modifies fatty acid composition of mouse adipose tissue, brain, and liver. Lipids, 32: 965-969.

van de Loo, F.J., Broun, P., Turner, S. and Somerville, C. (1995). An oleate 12-hydroxylase from *Ricinus communis* L. is a fatty acyl desaturase homolog. Proccedings of the National Academy of Sciences of the United States of America, 92: 6743-6747.

Lu, L.C., Bayon de Noyer, S., Hobbs, H.D., Kang, J., Wen, Y., Krachtus, D. and Hills, J.M. (2003) Expression pattern of diacylglycerol acytransferase-1, an enzyme involved in triacylglycerol biosynthesis, in *Arabidopsis thaliana*. Plant Molecular Biology, 52: 31-41.

Mantel, P.G. and Nisbet, L.J. (1976) Differentiation of *Claviceps purpurea* in anexic cultures. Journal of General Micribiology, 93: 321-334.

Meesapyodsuk, D. and Qiu, X. (2008). An oleate hydroxylase from the fungus *Claviceps purpurea*: cloning, functional analysis, and expression in *Arabidopsis*. Plant Physiology, 147: 1325-1333.

Meesapyodsuk, D., Reed, W. D., Covello, S. P. and Xiao, Q. (2007) Primary structure, regioselectivity, and evolution of the membrane-bound fatty acid desaturases of *Claviceps purpurea*. The Journal of Biological Chemistry, 282: 20191-20199.

Morris, L.J. and Hall, S.W. (1966). The structure of the glycerides of ergot oil. Lipids, 1: 188-196.

Morris, L.J., Hall, S.W. and James, A.T. (1966). The biosynthesis of ricinoleic acid by *Claviceps purpurea*. Biochemical Journal, 100: 29c-30c.

Morris, J.L. and Wharry, D.M. (1966). Naturally occurring epoxy acids. IV. The absolute optical configuration of vernolic acid. Lipids, 1: 41-46.

Nagiec, M.M., Wells, G.B., Lester, R.L. and Dickson, R.C. (1993) A suppressor gene that enables *Saccharomyces cerevisiae* to grow without making sphingolipids encodes a protein that resembles an Escherichia coli fatty acyltransferase. Journal of Biological Chemistry, 268:22156–22163

Noda, N., Umebayashi, K., Nakatani, T., Miyahara, K. and Ishiyama, K. (2005). Isolation and characterization of some hydroxy fatty and phosphoric acid esters of 10-hydroxy-2-decenoic acid from the royal jelly of honeybees (*Apis melifera*). Lipids, 40: 833-838.

Nykiforuk, L.C., Furukawa-Stoffer, L.T., Huff, W.P., Sarna, M., Laroche, A., Moloney, M.M. and Weselake, J.R. (2002) Characterization of cDNA encoding diacylglycerol acyltransferase from cultures of *Brassica napus* and sucrose-mediated induction of enzyme biosynthesis. Biochimica et Biophysica Acta, 1580: 95-109.

O'Donnell, K., Kistler, H.C., TackB.K. and Casper, H.H. (2000). Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. Proceedings of the National Academy of Science of the United States of America, 97: 7905-7910.

Oelkers, P., Cromley, D., Padamsee, M., Billheimer, J.D. and Sturley, S.L. (2002) The *DGA1* gene determines a second triglyceride synthetic pathway in yeast. The Journal of Biological Chemistry, 277: 8877-8881.

Paul, K.S., Jiang, D., Morita, Y.S. and Englund, P.T. (2001). Fatty acid synthesis in African trypanosomes: a solution to the myristate mystery. Trends in Parasitology, 17: 381-387.

Pazoutova, S. (2002) The evolutionary strategy of *Claviceps*. New York, Basel, pp. 329-354

Pazoutova, S., Olsovska, J., Linka, M., Kolínská, R. and Flieger, M. (2000). Chemoraces and habitat specialization of Claviceps purpurea populations. Applied and Environmental Microbiology, 66: 5419-5425

Pillai, M.G., Certik, M., Nakahara, T. and Kamisaka, Y. (1998) Characterization of triacylglycerol biosynthesis in subcellular fractions of an oleaginous fungus, *Mortierella ramanniana* var. *angulispora*. Biochemica et Biophysica Acta, 1393: 128-136.

Pollard, M., Beisson, F., Li, Y. and Ohlrogge, J.B. (2008). Building lipid barriers: biosynthesis of cutin and suberin. Trends in Plant Science, 13: 236-246.

Polokoff, M.A. and Bell, R.M. (1980). Solubilization, partial purification and characterization of rat liver microsomal diacylglycerol acyltransferase. Biochimica et Biophysica Acta, 618: 129-142.

Qiu, X., Reed, D.W., Hong, H., MacKenzie, S.L. and Covello, P.S. (2001). Identification and analysis of a gene from *Calendula officinalis* encoding a fatty acid conjugase. Plant Physiology, 125: 847-855.

Rawsthorne, S. (2002). Carbon flux and fatty acid synthesis in plants. Progress in Lipids Research, 41: 182-196.

Reed, W.D., Schafer, A.U. and Covello, S.P. (2000) Characterization of the Brassica napus extraplastidial linoleate desaturase by expression in *Saccharomyces cerevisiae*. Plant Physiology, 122: 715-720.

Reis, M.G., de Faria, A.D., Alves dos Santos, I., Amaral, M.C.E. and Marsaioli, A.J. (2007). Byrsonic acid – the clue to floral mimicry involving oil-producing flowers and oil-collecting bees. Journal of Chemical Ecology, 33: 1573-1561.

Rock, C.O. and Cronan, J.E. (1996). *Escherichia coli* as a model for the regulation of dissociable (type II) fatty acid biosynthesis. Biochimica et Biophysica Acta, 1302: 1-16.

Romanus, K., vanNeer, W., Marinova, E., Verbeke, K., Luypaerts, A., Accardo, S., Hermans, I., Jacobs, P., de Vos, D. and Waelkens, M. (2008). Brassicaceae seed oil identified as illuminant in Nilotic shells from the first millennium AD Coptic church in Bawit, Egypt. Analytical and Bioanalytical Chemistry, 390: 783-789.

Routaboul, J-M., Benning, C., Bechtold, N., Caboche, M. nd Lepiniec, L. (1999). The *TG1* locus of *Arabidopsis* encodes for a diacylglycerol acyltransferase. Plant Physiology and Biochemistry, 37: 831-840

Saha, S., Enugutii, B., Rajakumari, S. and Rajasekharan, R. (2006). Cytosolic triacylglycerol biosynthetic pathway in oilseeds. Molecular cloning and expression of peanut cytosolic diacylglycerol acyltransferase. Plant Physiology, 141: 1533-1543.

Sandager, L., Gustavsson, H.M., Stahl, U., Dahlqvist, A., Wiberg, E., Banas, A., Lenman, M., Ronne, H. and Stymne, S. (2001) Storage lipid synthesis is non-essential in yeast. The Journal of Biological Chemistr, 277: 6478-6482.

Shanklin, J. and Cahoon, E.B. (1998). Desaturation and related modifications of fatty acids. Annual Review of Plant Physiology, 49: 611-641.

Shockey, J.M., Gidda, S.K., Chapital, D.C., Kuan, J-C., Dhanoa, P.K., Bland, J.M., Rothstein, S.J., Mullen, R.T. and Dyer, J.M. (2006). Tung tree DGAT1 and DGAT2 have nonredundant functions in triacylglycerol biosynthesis and are localized to different subdomains of the endoplastic reticulum. The Plant Cell, 18: 2294-2313.

Simon, J.W. and Slabas, A.R. (1998). cDNA cloning of Brassica napus malonyl-CoA:ACP transacylase (MCAT) (FabD) and complementation of an *E. coli* MCAT mutant. FEBS Letters, 435: 204-206.

Smith, M.A. and Moon, H. (2003) Heterologous expression of a fatty acid hydroxylase gene in developing seeds of *Arabidopsis thaliana*. Planta, 217: 507-515

Smith, M.A., Jonsson, L., Styme, S. and Stobart, K. (1992). Evidence for cytochrome-b_5 as an electron-donor in ricinoleic acid biosynthesis in microsomal preparations from developing castor bean (*Ricinus-communis* L). Biochemical Journal, 287: 141-144.

Smith, S. (1994). The animal fatty acid synthase: one gene, one polypeptide, seven enzymes. The Journal of the Federation of American Societies for Experimental Biology, 8: 1248-1259.

Smith, S.J., Cases, S., Jensen, D.R., Chen, H.C., Sande, E., Tow, B., Sanan, D.A., Raber, J., Eckel, R.H. and Farese Jr, R.V. (2000). Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat. Nature Genetics, 25: 87-90.

Sorger, D. and Daum, G. (2003). Triacylglycerol biosynthesis in yeast. Applied Microbiology and Biotechnology, 61: 289-299.

Sorger, D. and Daum, G. (2002). Synthesis of triacylglycerols by the acylcoenzyme A:diacyl-glycerol acyltransferase Dga1p in lipid particles of the yeast *Saccharomyces cerevisiae*. Journal of Bacteriololy, 184: 519–524.

Stevens, A.E., Blakemore, J.A.E. and Reeves, C.J. (1997). Relationships amongst barley and oat infecting isolates of *Pyrenophora* spp. based on the internal transcribed spacer regions of ribosomal DNA. In: Hutchins, J.D., Reeves, J.C. (Eds.), *Seed Health Testing: progress towards the 21st century*. CAB International, Wallingford, p.139-145.

Stone, S.L., Levin, M.C., Zhou, P., Han, J., Walther, T.C. and Farese, R.V. Jr. (2009). The endoplasmic reticulum enzyme DGAT2 is found in mitochondria-associated membranes and has a mitochondrial targeting signal that promotes its association with mitochondria. The Journal of Biological Chemistry, 284: 5352-5361.

Stone, S.J., Levin, M. and Farese, R.V.Jr. (2006). Membrane topology and identification of key functional amino acid residues of murine acyl-CoA:diacylglycerol acyltransferase-2. Journal of Biological Chemistry, 281: 40273-40282.

Stone, S.J., Myers, H.M., Watkins, S.M., Brown, B.E., Feingold, K.R., Elias, P.M. and Farese, R.V. Jr. (2004). Lipopenia and skin barrier bnormalities in DGAT2-deficient mice. Journal of Biological Chemistry, 279: 11767-1776.

Suksamrarn, A., Bauprom, M., Udtip, S., Nuntawong, N., Haritakun, R. and Kanokmedhakul, S. (2005). Antimycobacteial and antiplasmodial unsaturated carboxylic acid from the twigs of *Scleropyrum wallichianum*. Chemical & Pharmaceutical Bulletin, 53: 1327-1329.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S. and Fisher, M.C. (2000). Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology, 31: 21-32.

Tsukiboshi, T., Shimanuki, T. and Uematsu, T. (1999) *Claviceps sorghicola* sp. Nov., a destructive ergot pathogen of sorghum in Japan. Mycological Research, 261: 133-141

Tudzynski, P., Correia, T. and Keller, U. (2001). Biotechnology and genetics of ergot alkaloids. Applied Microbiology and Biotechnology, 57: 593-605.

Tudzynski, P. and Scheffer, J. (2004). Claviceps purpurea: molecular aspects of a unique pathogenic lifestyle. Molecular Plant Pathology, 5: 377-388.

Tzen, J.T.C., Cao, Y., Laurent, P., Ratnayake, C. and Huang, A.H.C. (1993). Lipids, proteins, and structure of seed oil bodies from diverse species. Plant Physiology, 101: 267-276.

Voelker, D.R. (2000) Interorganelle transport of aminoglycerophospholipids. Biochimica et Biophysica Acta, 1486: 97–107

.

Waterman, I.J., Prise, N.T. and Zammit, V.A. (2002) Distinct ontogenic patterns of overt and DGAT activities of rat liver microsomes. Journal of Lipids Research, 43: 1555-1562.

Weiss, S.B., Kennedy, E.P. and Kiyasu, E.J. (1960). The enzymatic synthesis of triglycerides. Journal of Biological Chemistry, 235: 40–44.

Weselake, l.R., Kazala, E.C., Cianflone, K., Boehr, D.D., Middleton, K.C., Rennie, D.C., Laroche, A. and Recnik, I. (2000). Human acylation stimulating protein

enhances triacylglycerol biosynthesis in plant microsomes. FEBS Letters, 480: 189-192.

White, T.J., T. Bruns, S. Lee, and J. Taylor. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* Innis M.A., D.H. Gelfand, J.J. Sninsky, and T.J. White (Eds.) PCR protocols: a guide to methods and applications. p.315-322. San Diego, California: Academic Press Inc.

Wyatt, C.J., Pereira, L.R. and Day, E.A. (1967) The lactone precursor in fresh milk fat: Identification of the hydroxy fatty acids. Lipids, 2: 208-211.

Yen, C.E., Monetti, M., Burri, B.J. and Farese Jr, R.V. (2005). The triacylglycerol synthesis enzyme DGAT1 also catalyzes the synthesis of diacylglycerols, wax, and retinyl esters. Journal of Lipids Research, 46: 1502-1511.

Yu, K., McCracken, C.T., Runzhi Li and Hildebrant, D.F. (2006). Diacylglycerol from *Vernonia* and *Stokesia* prefer substrates with vernolic acid. Lipids, 41: 557-566.

Zhang, Y.M., Rao, M.S., Heath, R.J., Price, A.C., Olson, A.J., Rock, C.O. and White, S.W. (2001). Identification and analysis of the acyl carrier protein (ACP) docking site o β-ketoacyl-ACP sunthase III. Journal of Biological Chemistry, 276: 8231-8238.

Zheng, Z. and Zou, J. (2001). The initial step of the glycerolipid pathway: identification of glycerol 3 phosphate/dihydroxyacetone phosphate dual substrate acyltransferases in *Saccharomyces cerevisiae*. Journal of Biological Chemistry, 276:41710–41716

Zgoda, J, R., Freyer, A.J., Killmer, L.B. and Porter, L.R. (2001). Polyacetilene carboxylic acids from *Mitrephora celebica*. Journal of Natural Products, 46: 1348-1349.

Zhou, Z.Y., Wang, F., Tang, J.G., Fang, L.Z., Dong, Z.J. and Liu, J.K. (2008). Gallicynoic acids A-I, acetylenic acids from the basidiomycetes *Coriolopsis gallica*. Journal of Natural Products, 71: 223-226.

Zou, J., Wei, Y., Jako, C., Kumar, A., Selvaraj, G. And Taylor, D.C. (1999) The Arabidopsis thaliana TG1 mutant has a mutation in a diacylglycarol acyltransferase gene. The Plant Journal, 19: 645-653.